Structure-function studies of G protein-coupled receptors with targeted mutagenesis

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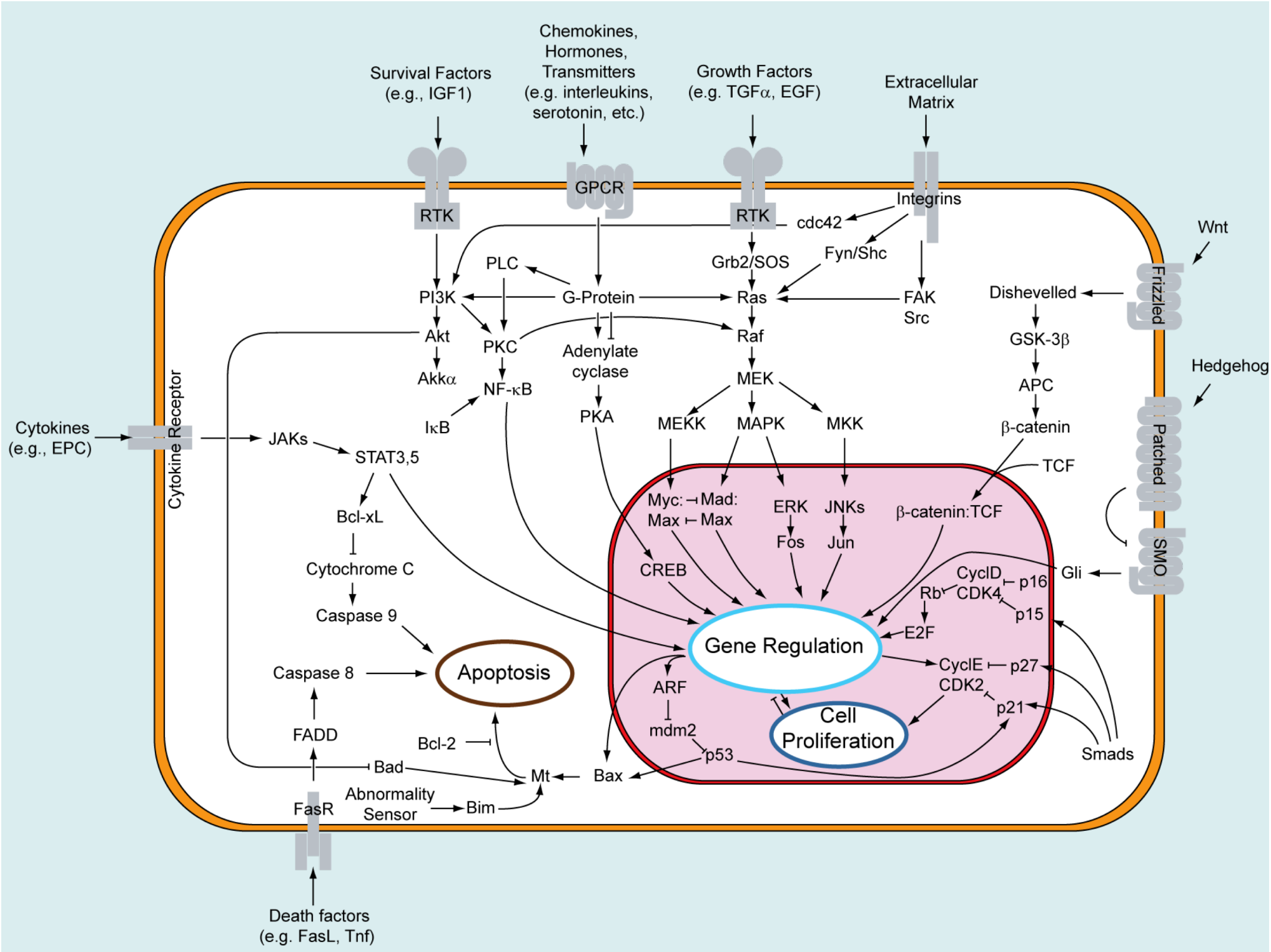
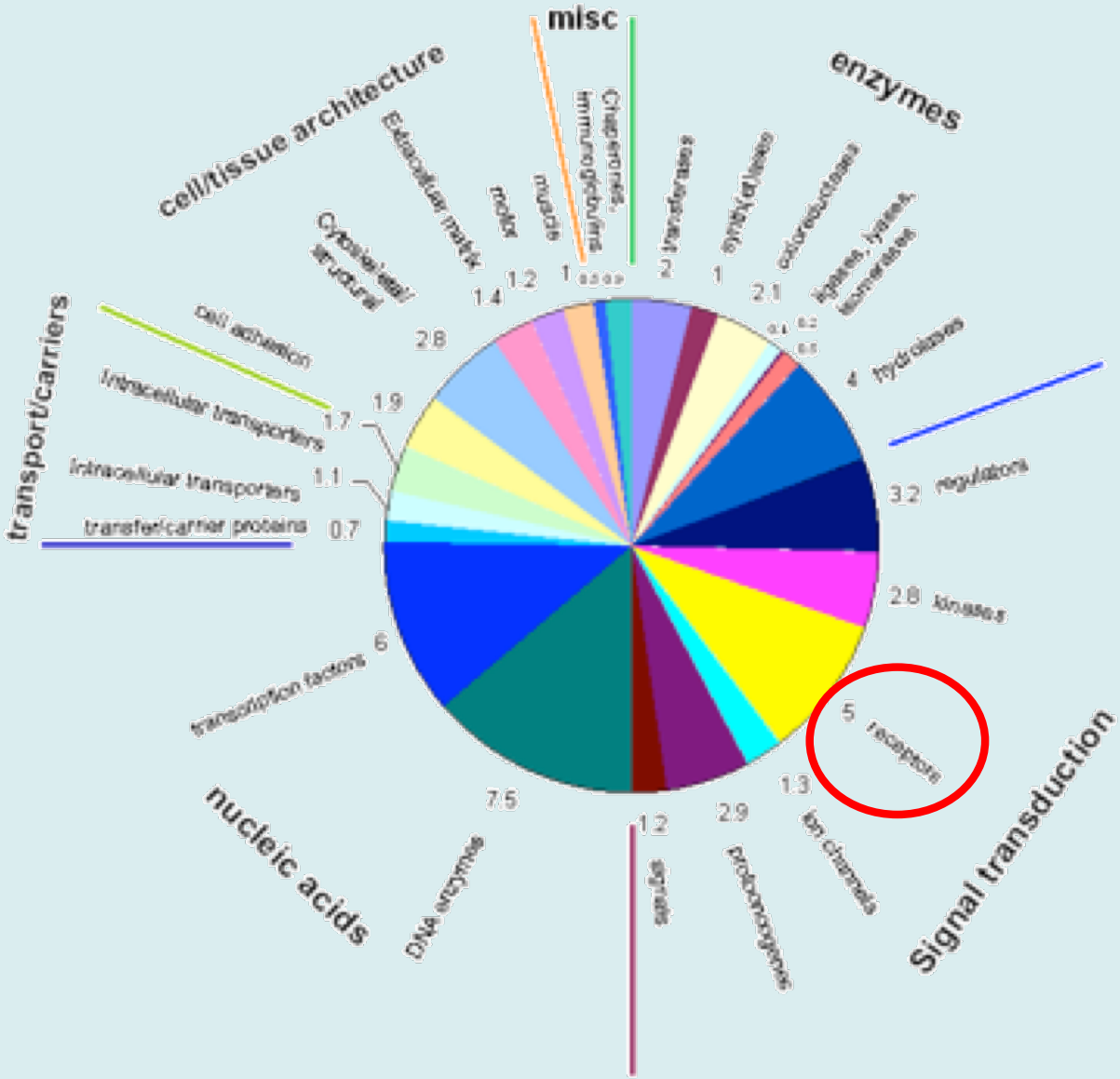
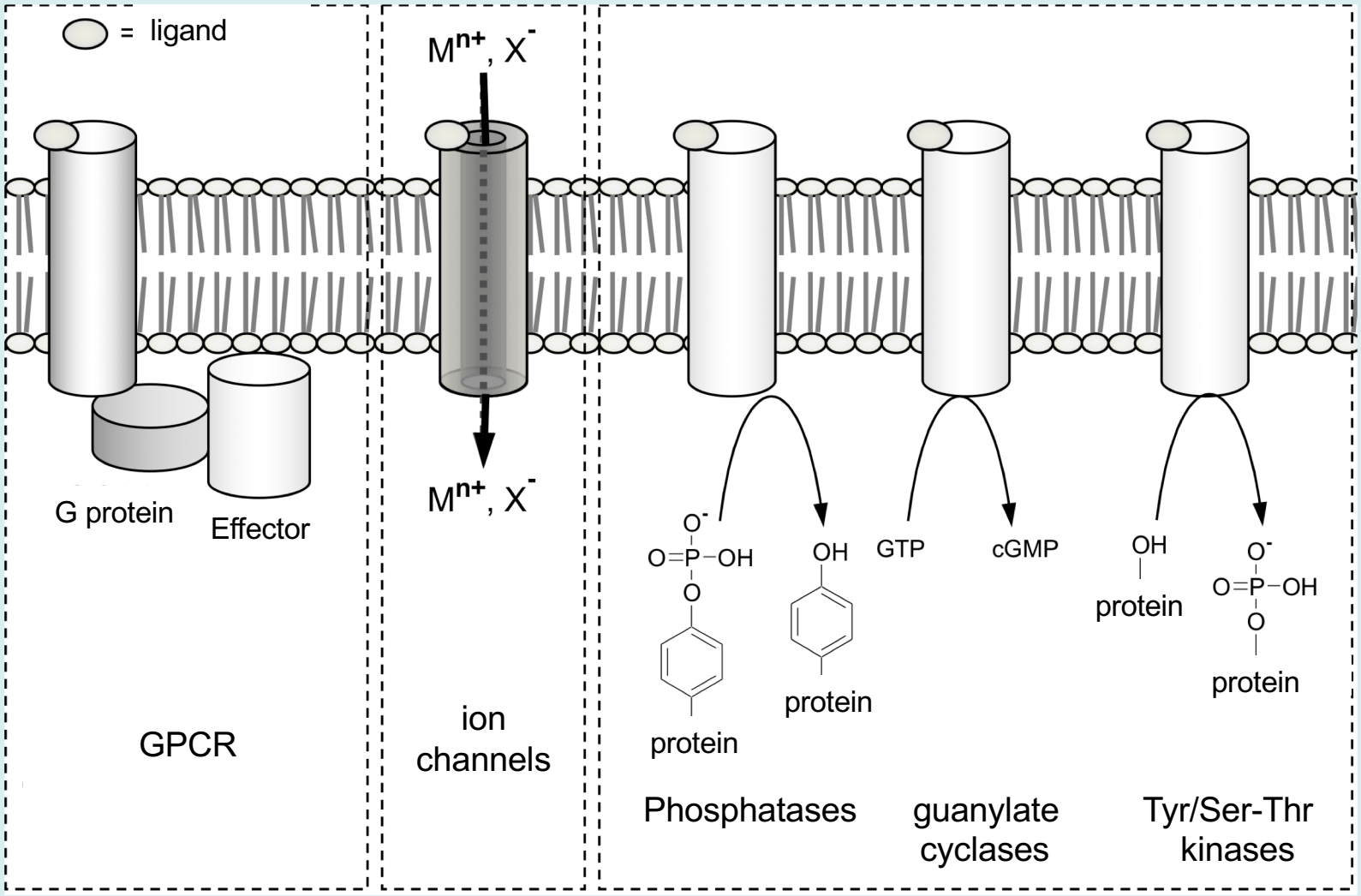
KÉM/224 PhD course 2019


Survival Factors
(e.g., IGF1)
Chemokines,
Hormones,
Transmitters
(e.g. interleukins,
serotonin, etc.)
Growth Factors
(e.g. TGFα, EGF)
Extracellular
Matrix
RTK
GPCR
RTK
Integrins
cdc42
Fyn/Shc
Grb2/SOS
PLC
PI3K
G-Protein
Ras
FAK
Src
Wnt
Frizzled
Dishevelled
GSK-3β
APC
β-catenin
Hedgehog
Patched
SMO
Akt
PKC
Adenylate
cyclase
Raf
Akkα
NF-κB
IκB
PKA
MEK
MEKK
MAPK
MKK
Cytokines
(e.g., EPC)
Cytokine Receptor
JAKs
STAT3,5
TCF
Bcl-xL
Myc: ⊣ Mad:
Max ⊢ Max
ERK
JNKs
β-catenin:TCF
Fos
Jun
Cytochrome C
CREB
CyclD
p16
Rb
CDK4
p15
Gli
Caspase 9
E2F
Gene Regulation
Caspase 8
Apoptosis
CyclE
p27
ARF
CDK2
p21
Cell
Proliferation
mdm2
FADD
Bcl-2
p53
Smads
Bad
Mt
Bax
FasR
Abnormality
Sensor
Bim
Death factors
(e.g. FasL, Tnf)

Receptors in the Human Genome



Main plasma membrane receptor types



G protein-coupled receptors (GPCR)
– why are they so interesting and important?

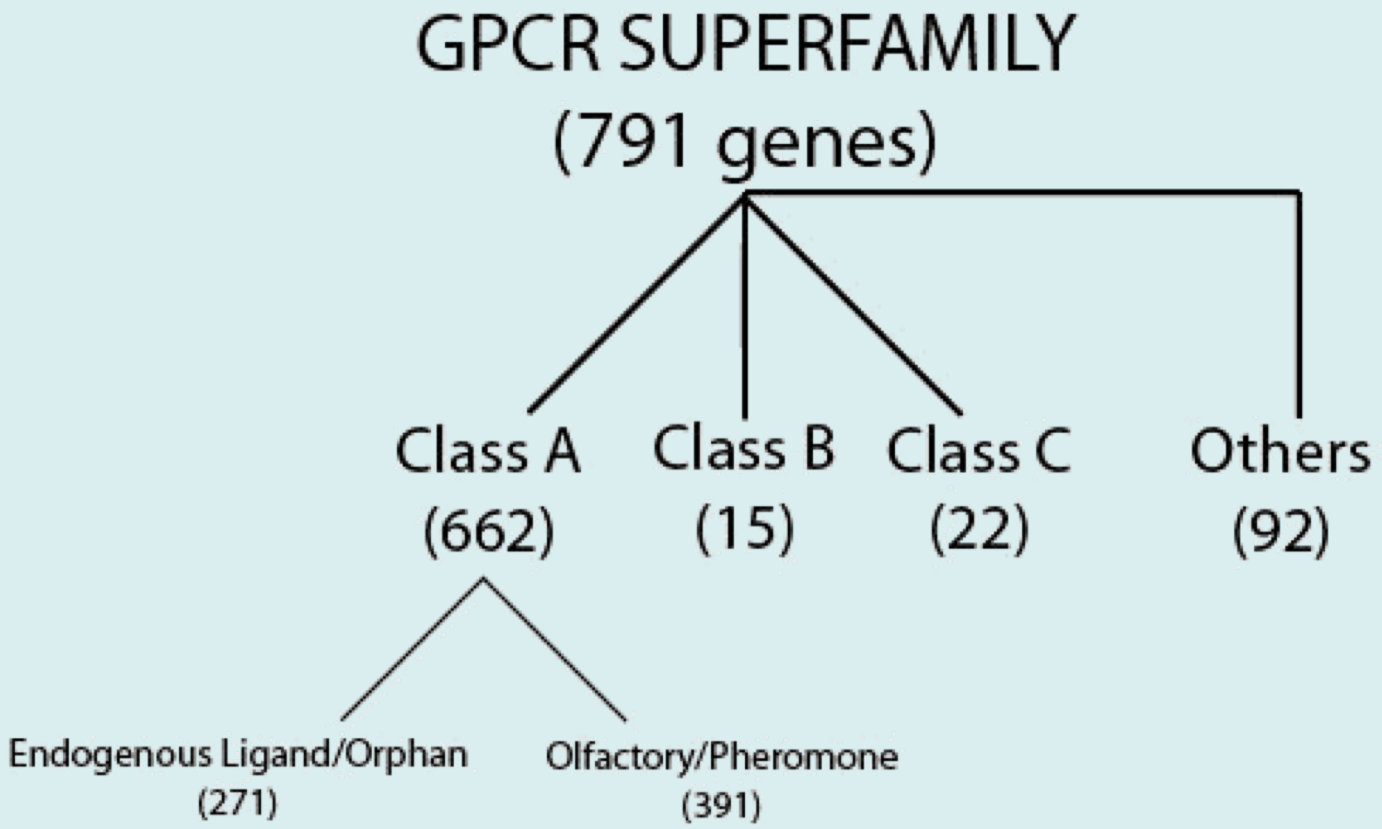
- GPCR signaling is the primary mechanism by which cells sense changes in their environment (light, odor, taste, pH, Ca^{2+}, hormones, lipid mediators, neurotransmitters, etc).
- The human genom (~20000 genes) includes 1543 non-redundant sequences coding signaling receptors, more than half of them are GPCR. ~400 of the ~800 GPCR are smell and taste receptors. ~300 genes encode GPCR for endogenous ligands. Taken the splice variants, post-translational modifications and average expression profiles, these receptors could potentially respond to ~33000 different ligand combinations.
- GPCR affect every aspect of cell physiology - proliferation, differentiation, apoptosis, motility, cell-shape, Ca^{2+} homeostasis, cell-to-cell communication, etc.

G protein-coupled receptors (GPCR)
– why are they so interesting and important?

- From pharmacological point of view GPCR are the most important class of drug targets: ~50% of the currently available drugs act on GPCR and the majority (>50%) of new drug developments target GPCR
- Endogenous ligands are still not known for >100 GPCR (orphan receptors)
- The main difficulty for rational drug design is the lack of experimentally determined 3D structures

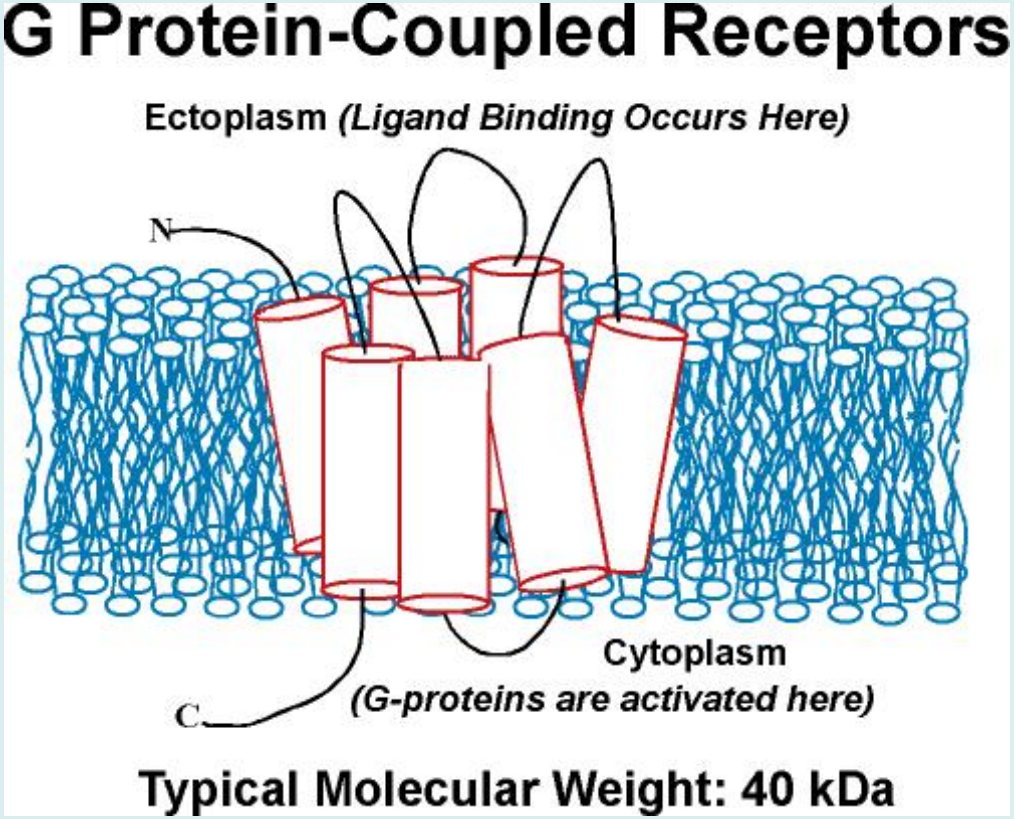
GPCR classification

Class A - Rhodopsin-like (most populous and diverse)
Class B - Secretins (glucagon, GnRH, PTH, CRH receptors)
Class C - Metabotropic glutamate / pheromone receptors
Class D - Fungal mating pheromone receptors
Class E - Frizzled/Smoothened, Wnt, Hedgehog



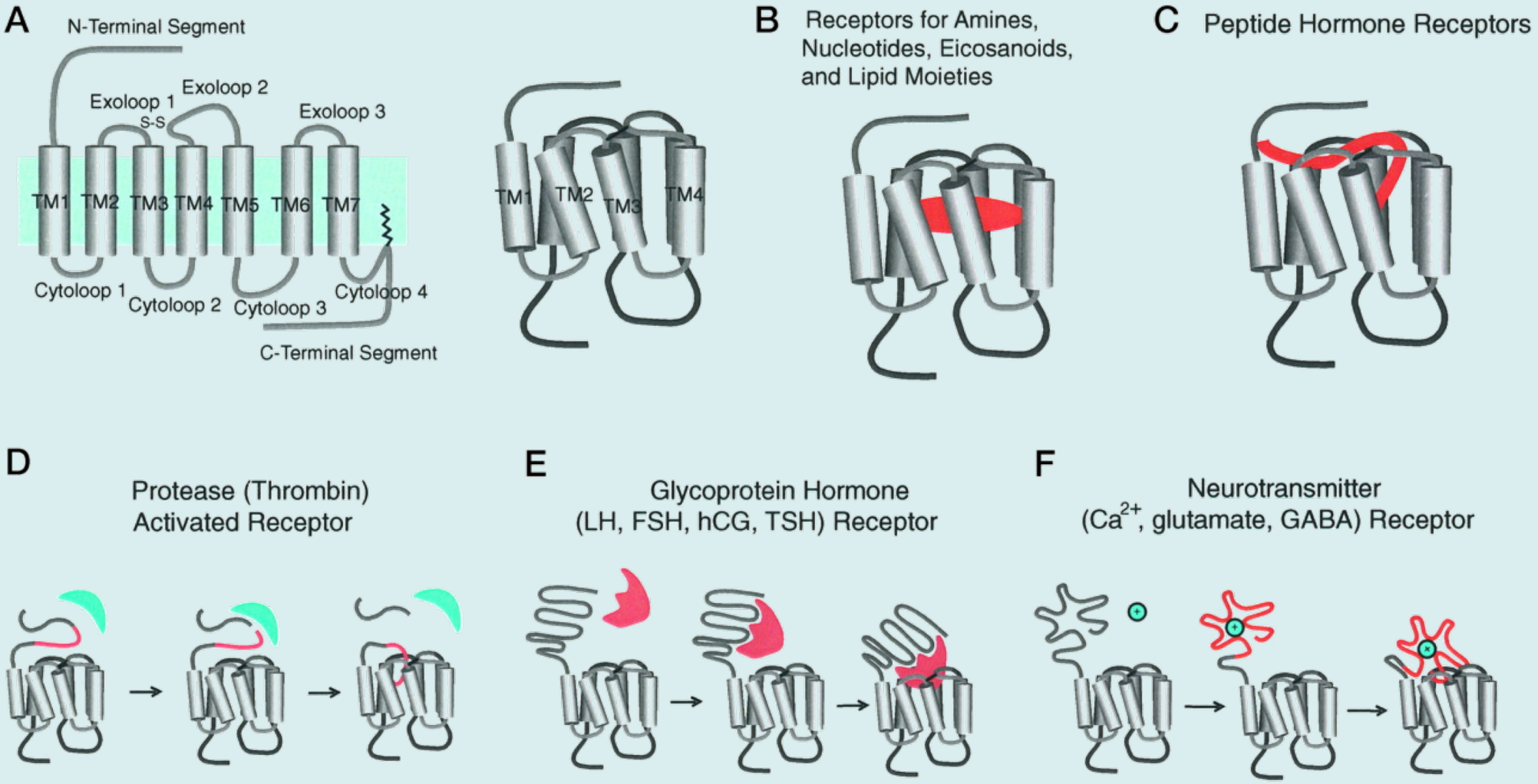
- Common structural features, activation and inactivation mechanisms
- Pharmacological description of GPCR function
- New concepts in GPCR function: dimerization, transactivation, allostery, biased agonism
- Gaining structural information utilizing homology modeling and ligand docking

Main structural features of GPCR

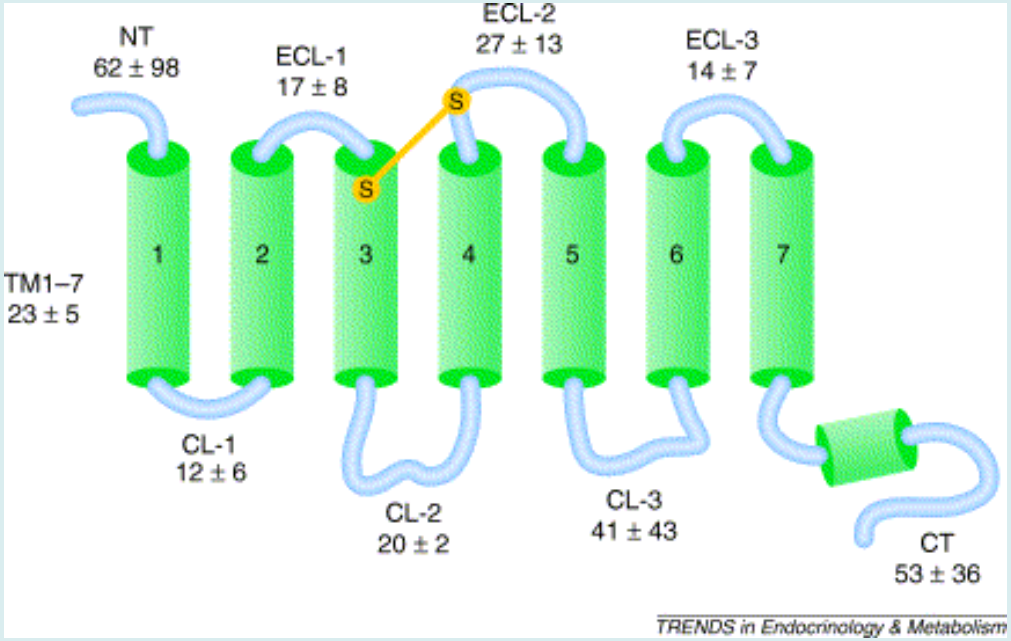


- Core of seven transmembrane α-helical bundle (GPCR also referred as 7TM receptors or heptahelical receptors)
- The antiparallel helices are linked by 3 intracellular and 3 extracellular loops
- C-terminal is positioned intracellularly.

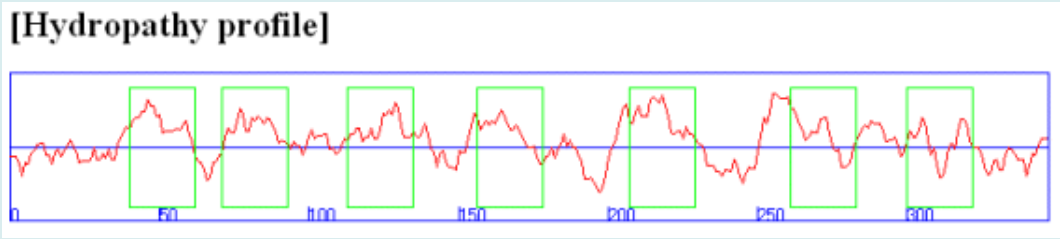
Common ligand binding modes of GPCR



Secondary structure of GPCR

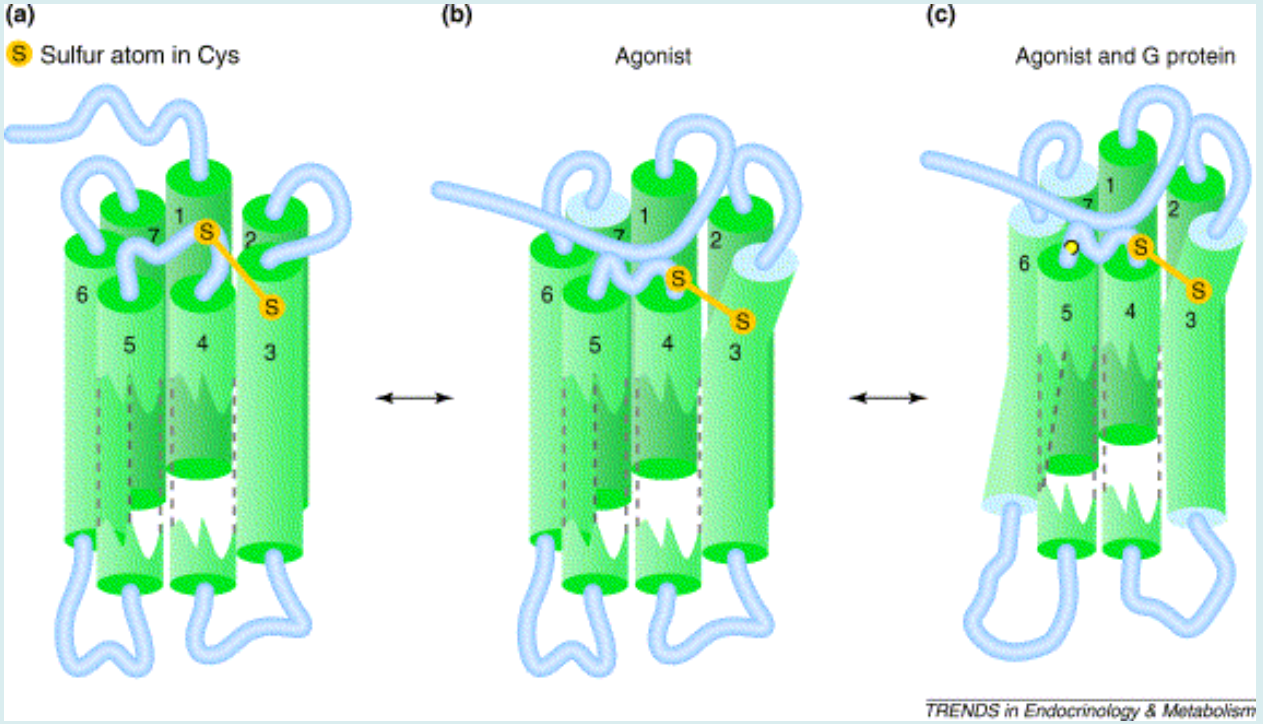


- 7TM structure identification is based on the presence of 7 stretches of ~25 hydrophobic residues - effective prediction algorithms are available.



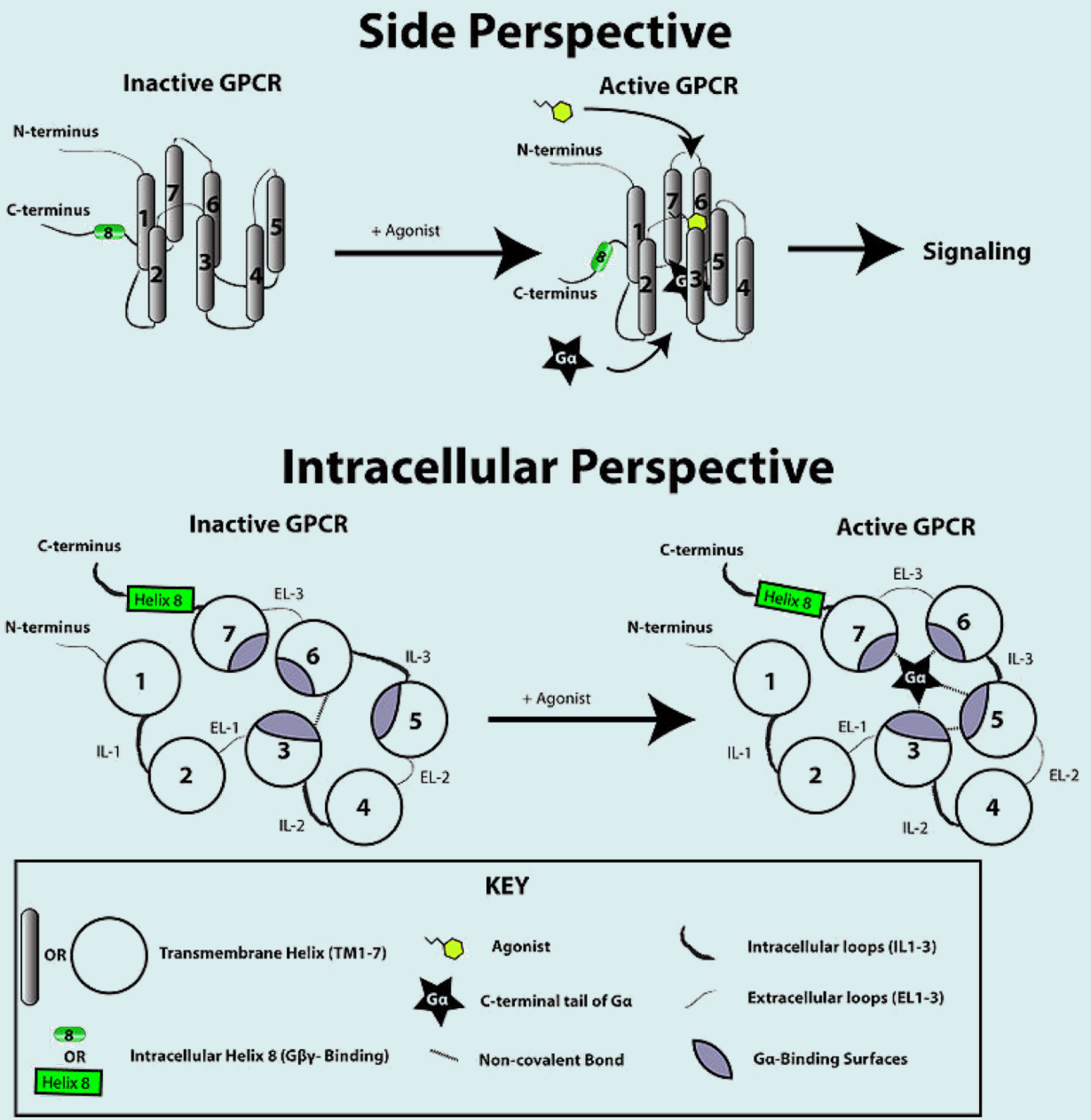
- Conserved features - S-S bound (92%), DRY motif in TM3, and NPxxY motif in TM7, the average length of TM helices and EC loops. N-terminal part is highly variable in length.

General GPCR activation mechanism - movements of TM helices and loops

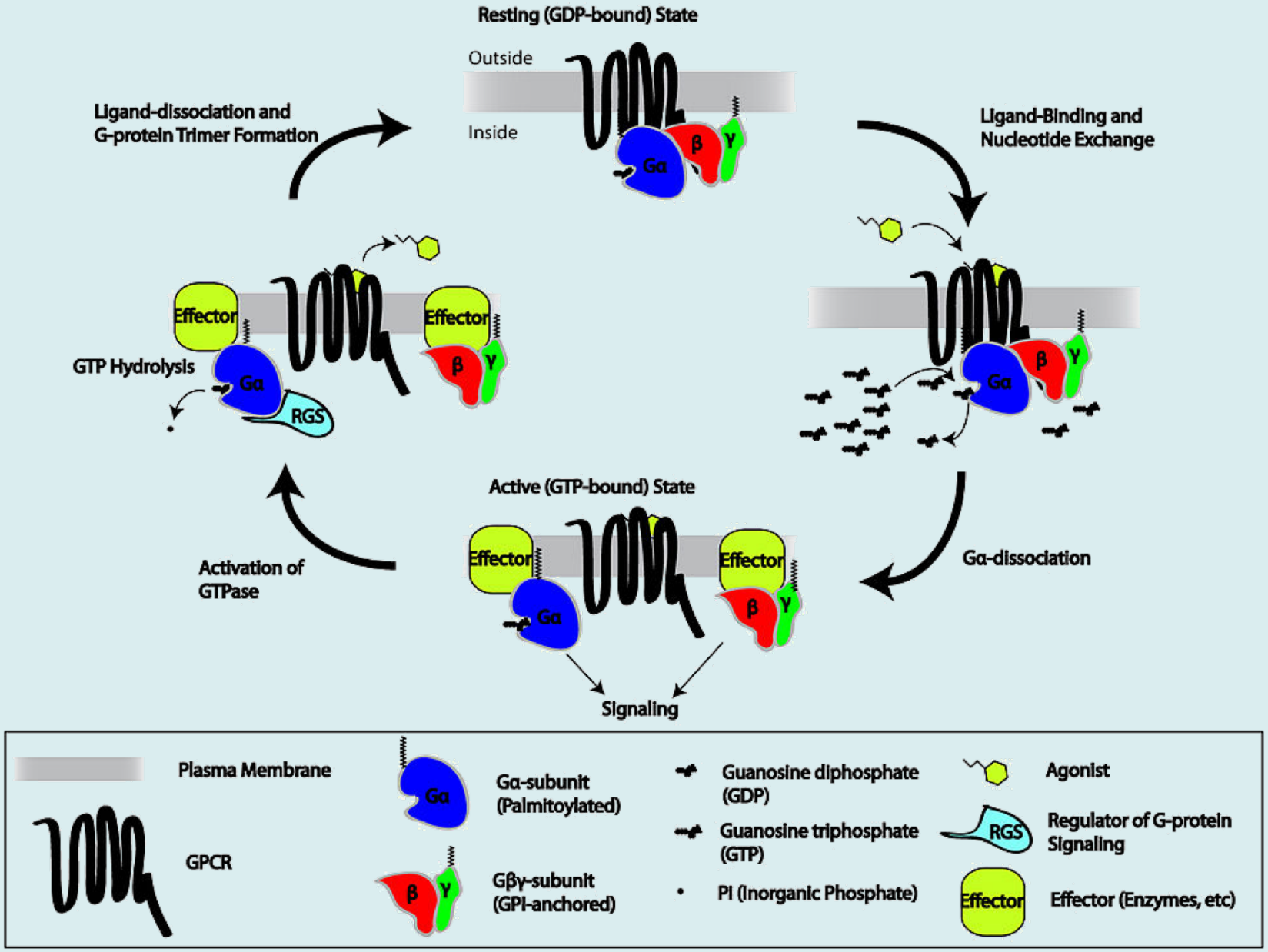


- Disruption of the salt bridge between TM3 and TM7.
- Large movements of TM6 and cytoplasmic loop IL3.
- Consequently, the inner faces of TM2, TM3, TM6, and TM7 become more exposed, while that of TM3 and TM4 become less exposed to the cytoplasm.

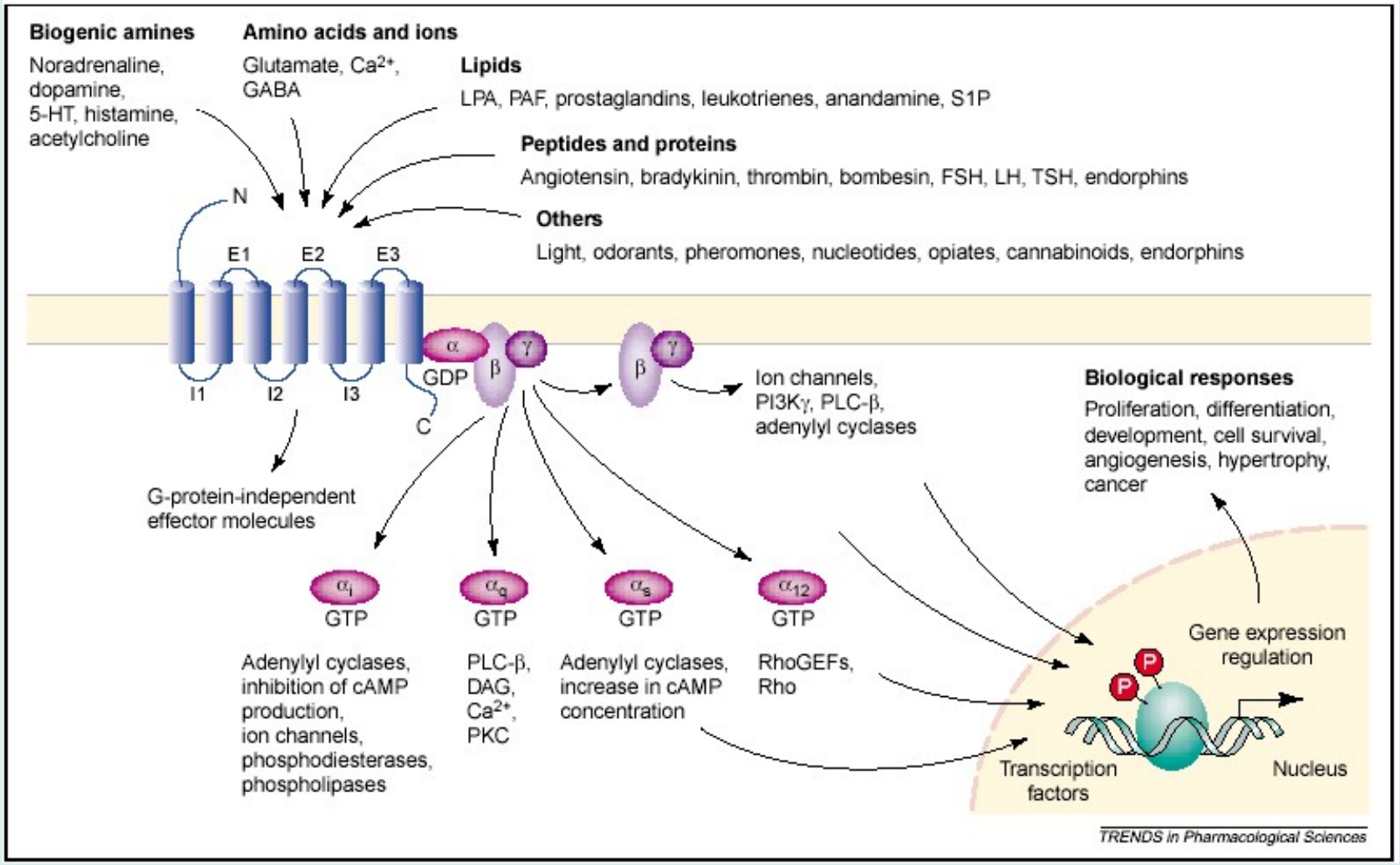
Inactive and active conformations of GPCR



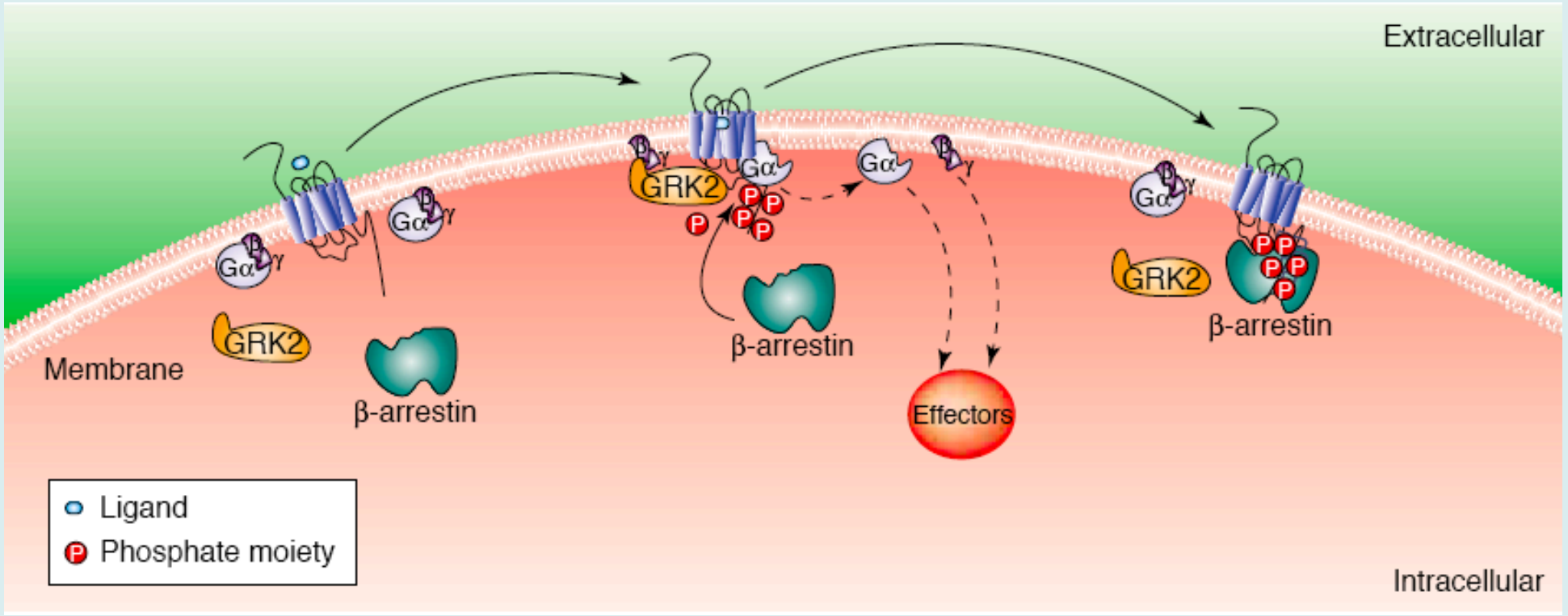
GPCR cycle


Resting (GDP-bound) State
Outside
Inside
Gα
β
γ
Ligand-Binding and
Nucleotide Exchange
Gα-dissociation
Active (GTP-bound) State
Effector
Effector
Signaling
Activation of
GTPase
GTP Hydrolysis
RGS
Ligand-dissociation and
G-protein Trimer Formation
Plasma Membrane
GPCR
Gα-subunit
(Palmitoylated)
Gβγ-subunit
(GPI-anchored)
Guanosine diphosphate
(GDP)
Guanosine triphosphate
(GTP)
PI (Inorganic Phosphate)
Agonist
Regulator of G-protein
Signaling
Effector (Enzymes, etc)

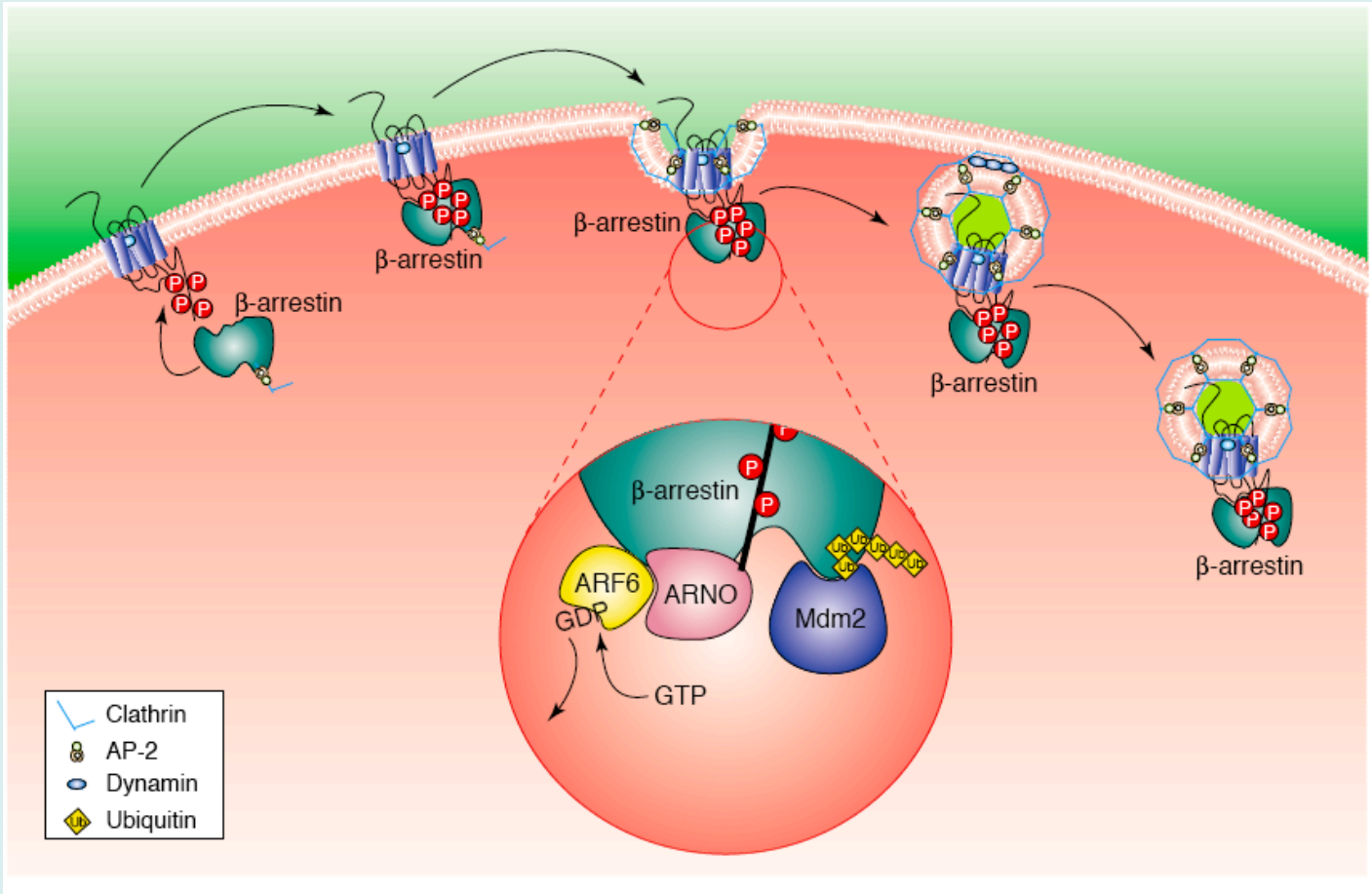
Overview of GPCR-activated signaling pathways



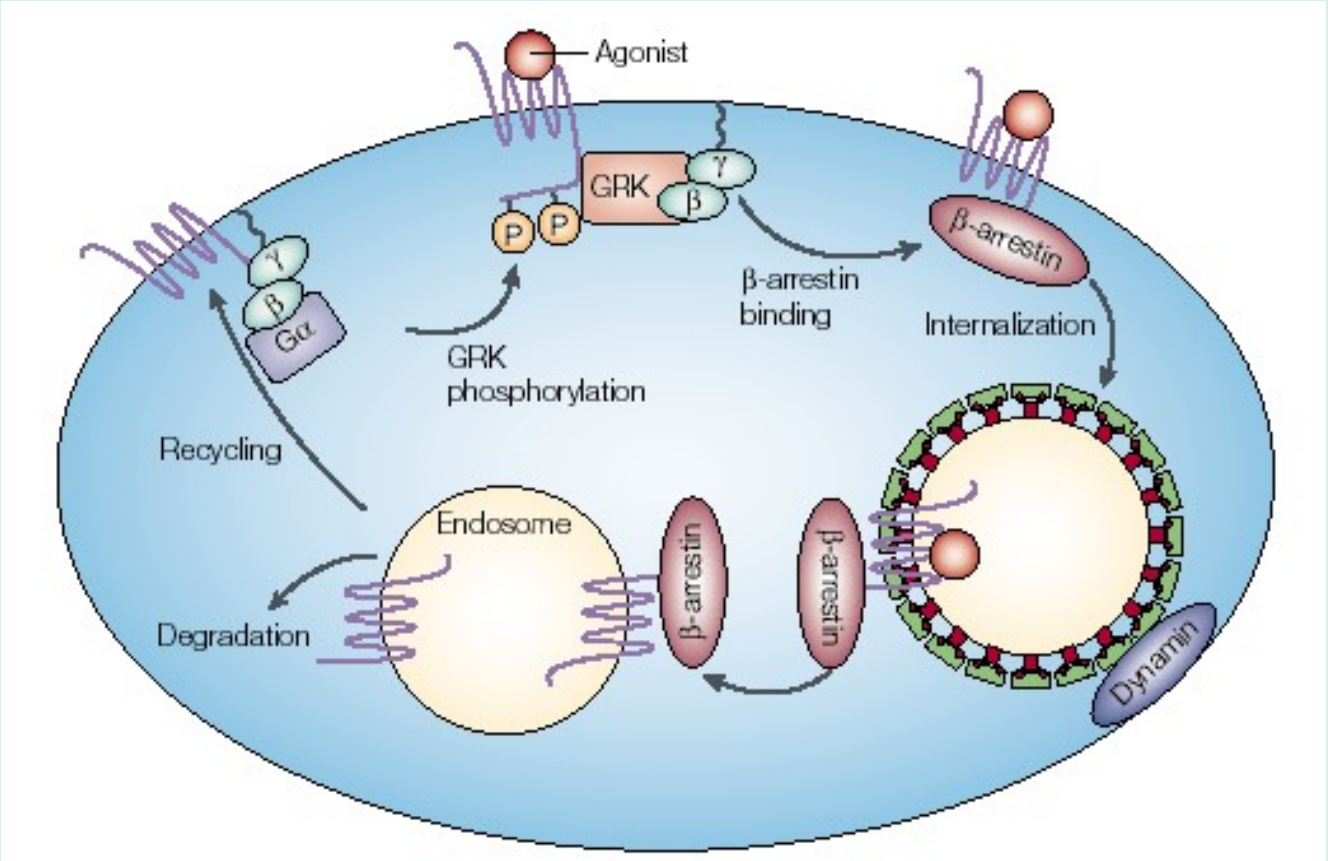
GPCR activation and inactivation mechanisms: G protein-activation followed by desensitization



Clatrin-dependent internalization of GPCR



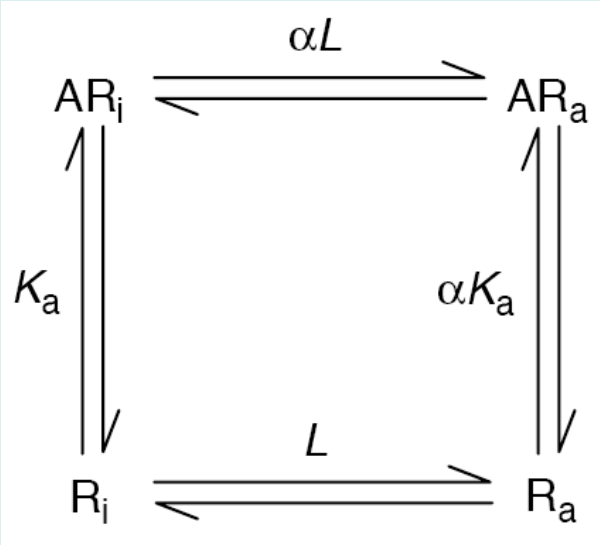
GPCR trafficking - receptor recycling



Activation of GPCR by an agonist leads to the dissociation of α and $\beta\gamma$ subunits. The free $\beta\gamma$ dimers recruit specific kinases (GRK) to the receptor, where they selectively phosphorylate agonist-occupied receptors. This, in turn, leads to the recruitment of β-arrestin to the receptor and targets the receptor-β-arrestin complex to clathrin-coated pits. The receptor is internalized into acidic endosomes and then either dephosphorylated and returned to the cell surface or degraded.

Pharmacological description of GPCR function

Two-state model of GPCR activation

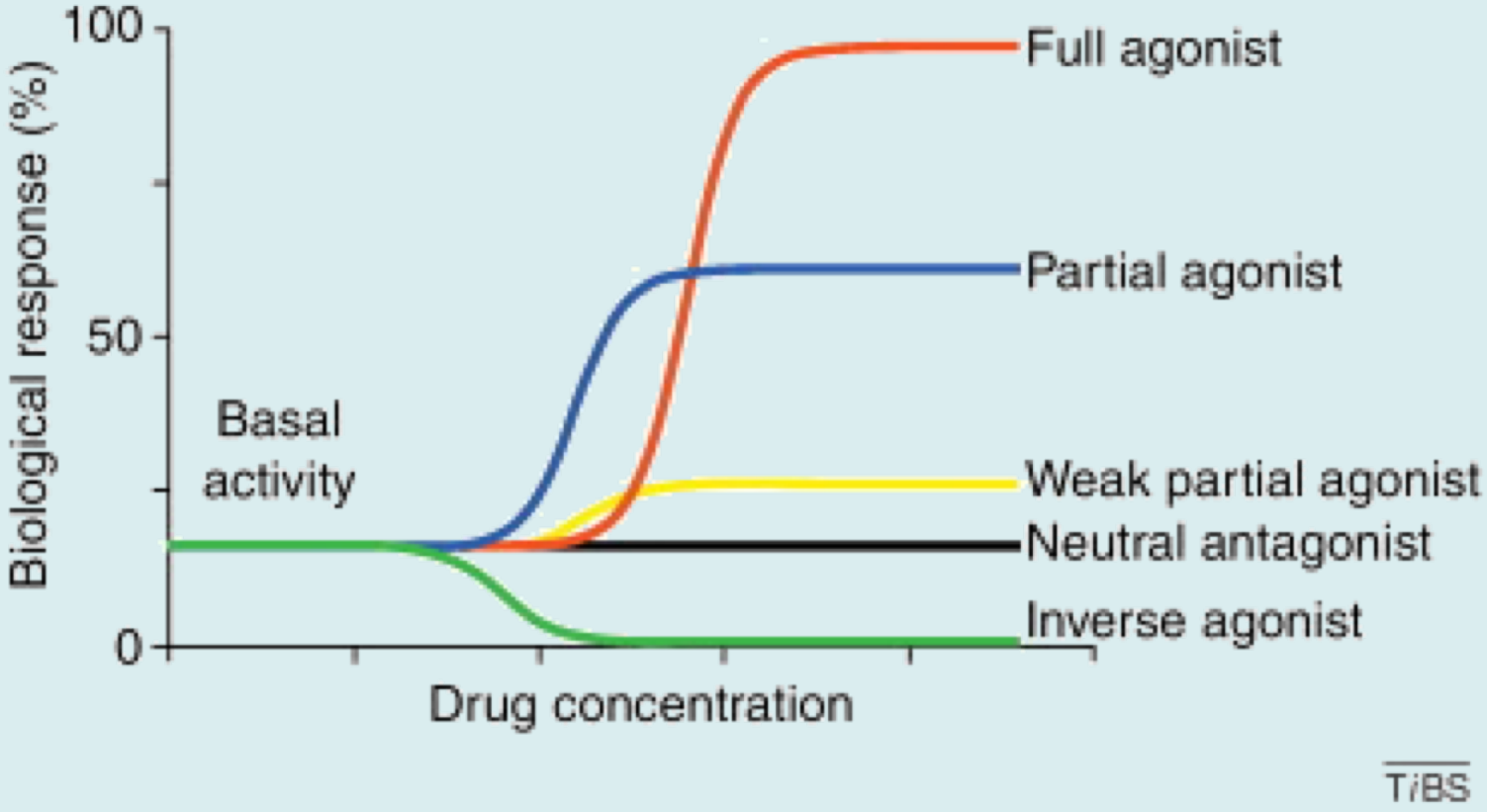


$$\rho = \frac{L(1+\alpha[A]/K_A)}{[A]/K_A(1+\alpha L)+L+1}$$

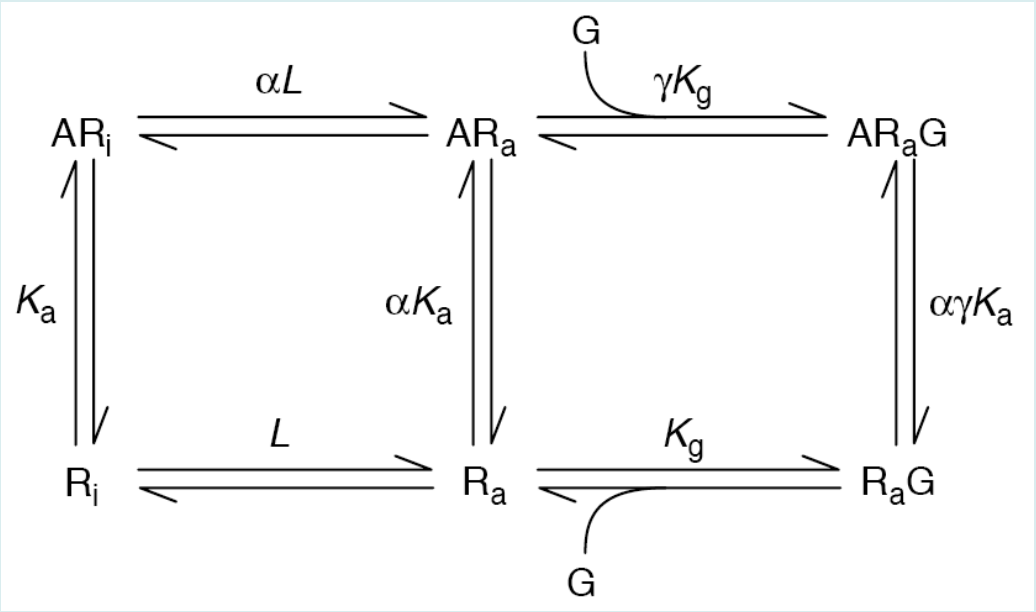
In the absence of ligand {i.e. [A] = 0}, $\rho_0 = L/(1 + L)$ and in the presence of a maximal concentration of ligand {i.e. saturating the receptors, where [A] ~∞}, $\rho_\infty = (\alpha L)/(1 + \alpha L)$. Therefore, the effect of a ligand on changing the ratio between the active and inactive states is given by the ratio $\rho_\infty/\rho_0 = \alpha(1 + L)/(1 + \alpha L)$. For example, if L=0.1, 9% of receptors would be in the active state in the absence of ligand. Addition of saturating concentration of ligand with α=5 would increase the ratio of active-state receptors to 33%.

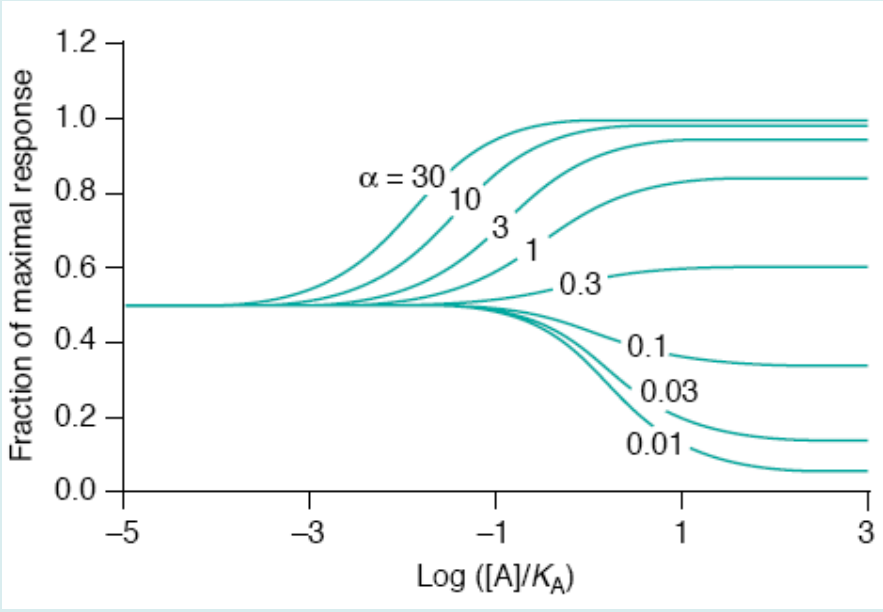
- Ligand A interacts with both the active (R_a) and inactive (R_i) conformers, thereby changing the equilibrium between the two forms
- If $\alpha>1$ the ligand is an agonist, it shifts the equilibrium toward R_a
- If $\alpha<1$ the ligand is an inverse agonist, it shifts the equilibrium toward R_i
- If $\alpha=1$ the ligand is a competitive antagonist, it does not change the equilibrium between R_a and R_i but decreases the number of free receptor conformers for other ligands to interact with

Two-state model of GPCR activation



Ternary complex model for GPCR activation

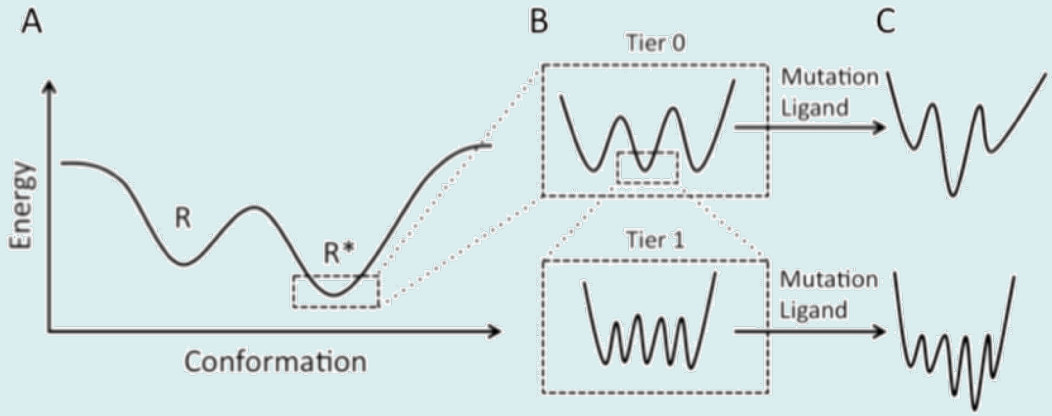




As can be seen from the dose-response curves, a ligand can be an agonist even with α=1! Similar curves are resulted when changing γ. Taken together, the ability of a ligand to increase or decrease the basal activity of the receptor depends on the distribution of affinities toward the different receptor conformational states. Chemically similar ligands can behave quite differently.

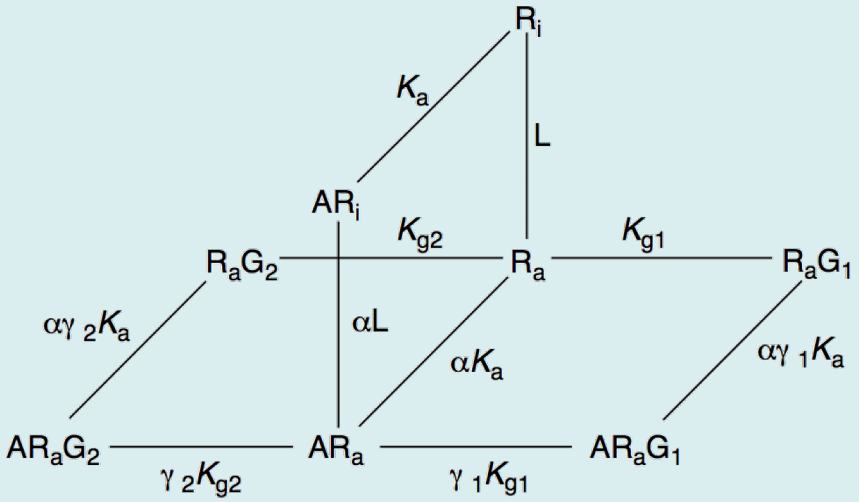
But GPCR activation is even more complex:

The number of activation states can be increased but there are experimental data just don't fit to such "discrete-states" models. The current understanding is that GPCR conformational changes reflect the dynamics/flexibility of the protein and several of these conformers can be regarded as active conformations.

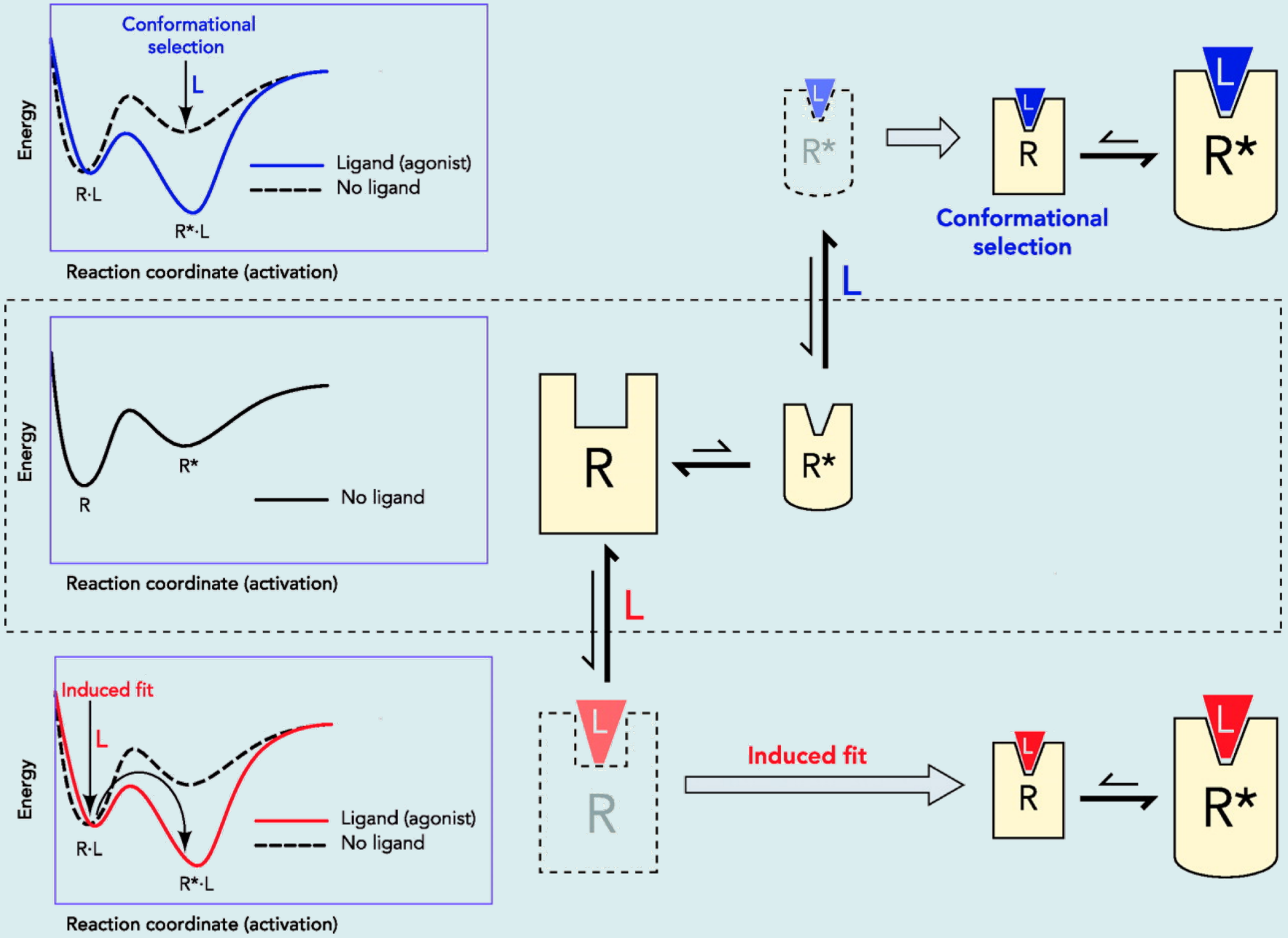


Conformational ensemble

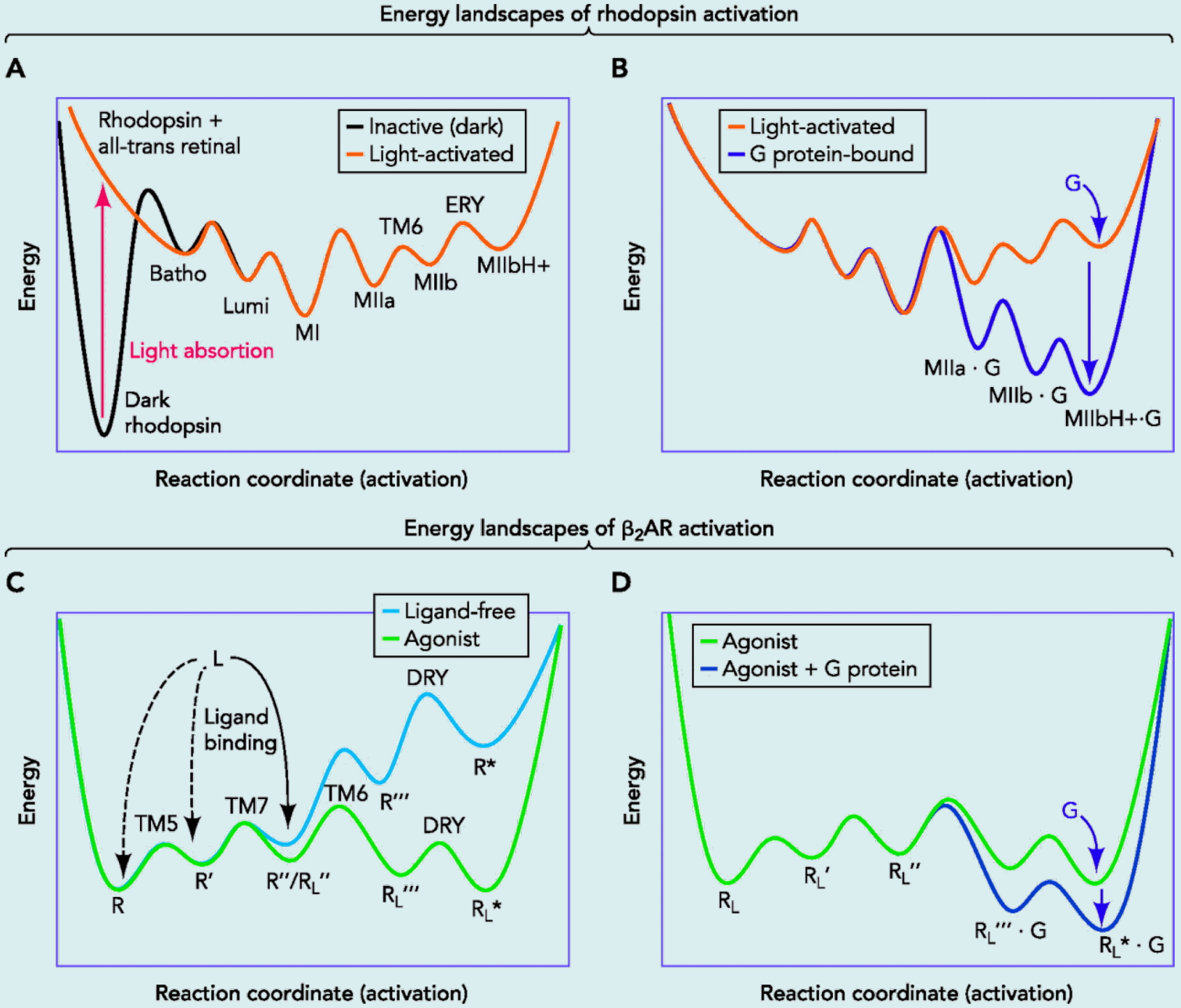
Different states



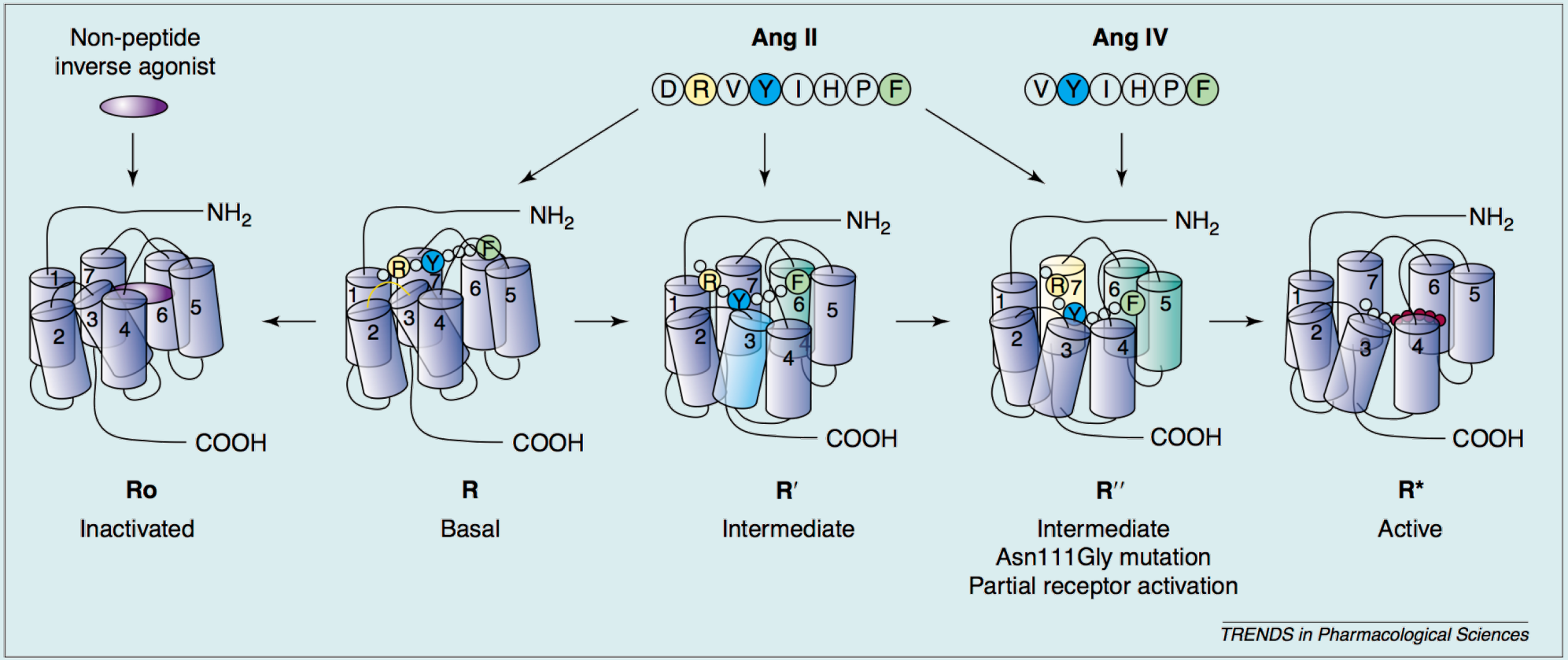
Conformational selection or induced fit?



Conformational selection or induced fit?



Conformational selection or induced fit?



Homodimerization

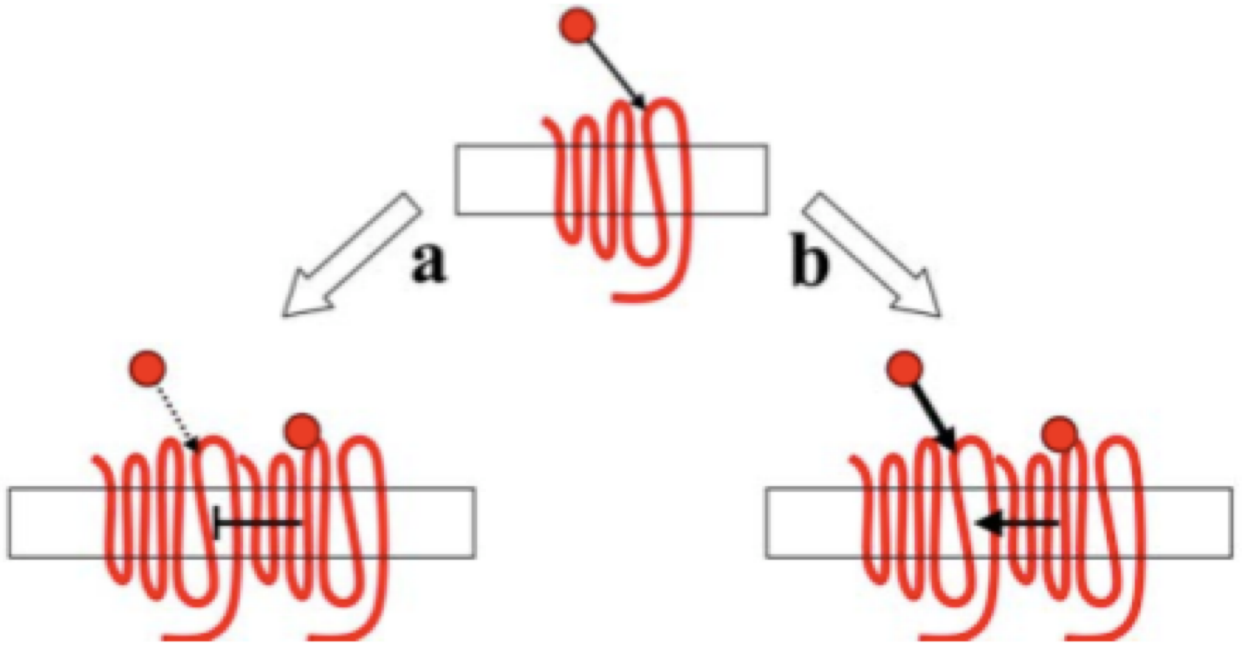


FIGURE 2. Intermolecular interactions in the receptor homodimer. The affinity of a given ligand for its receptor (central drawing) may be decreased (a) or increased (b) when the ligand already occupies the receptor partner (in the homodimer).

Heterodimerization

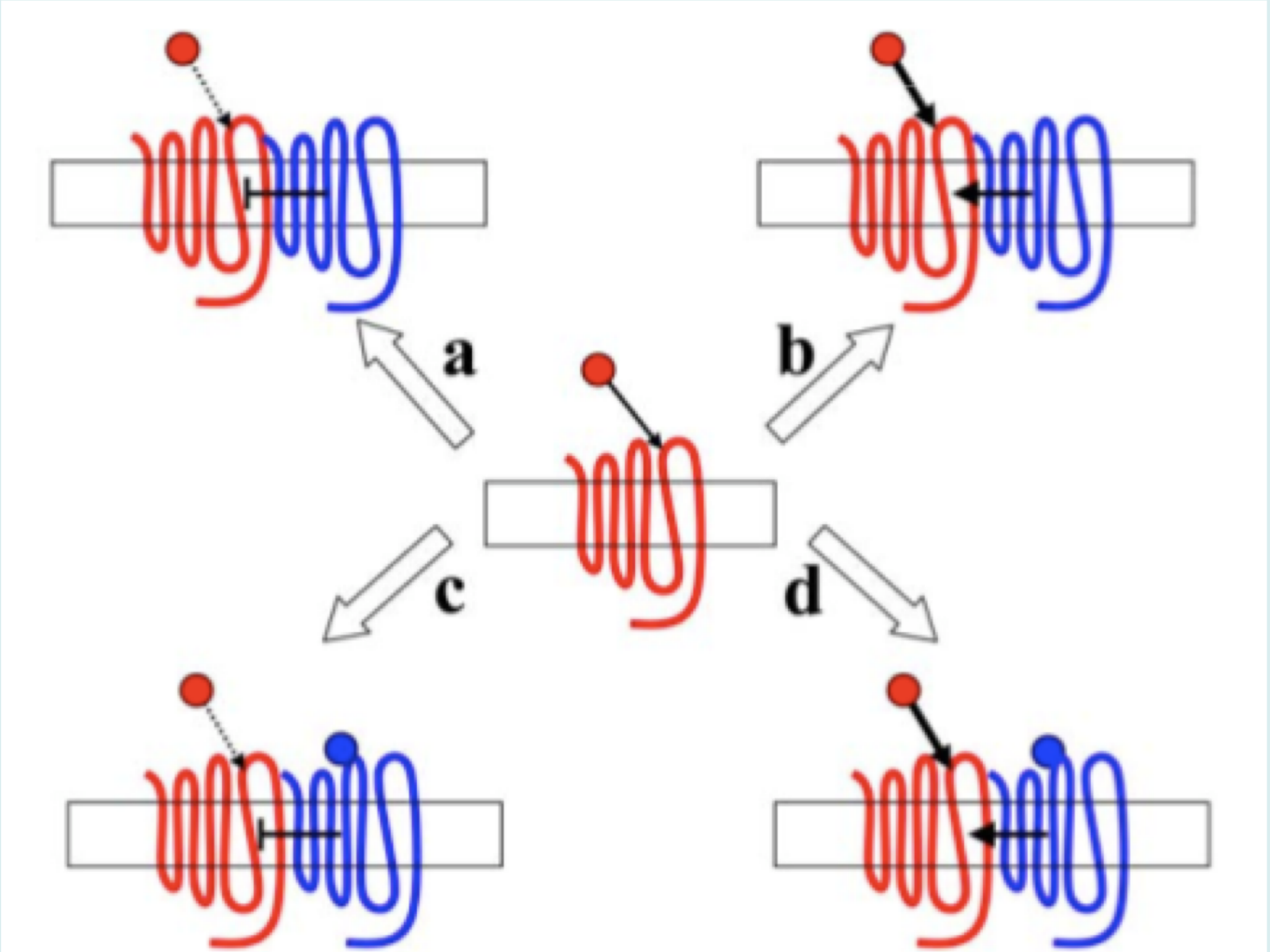
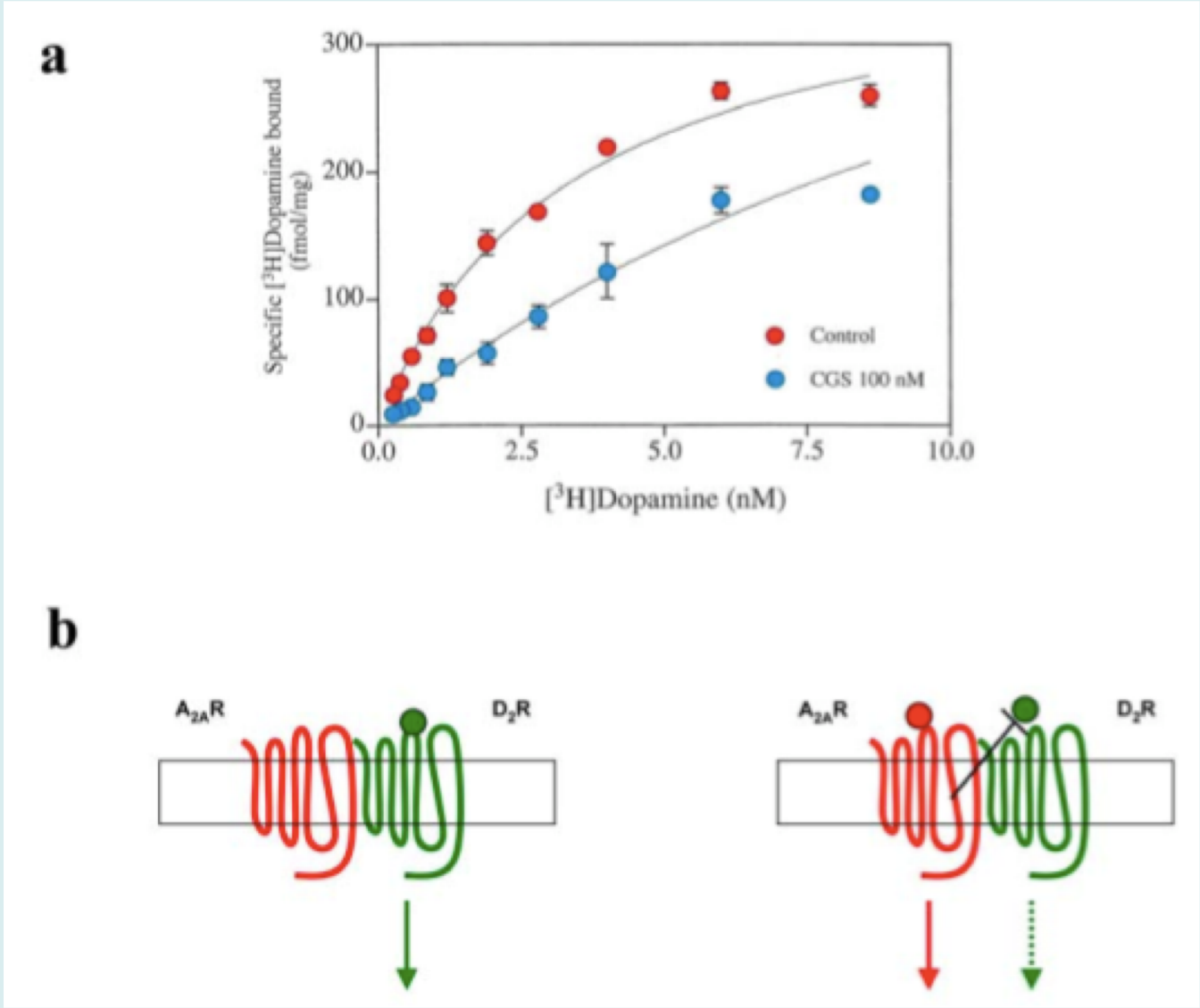
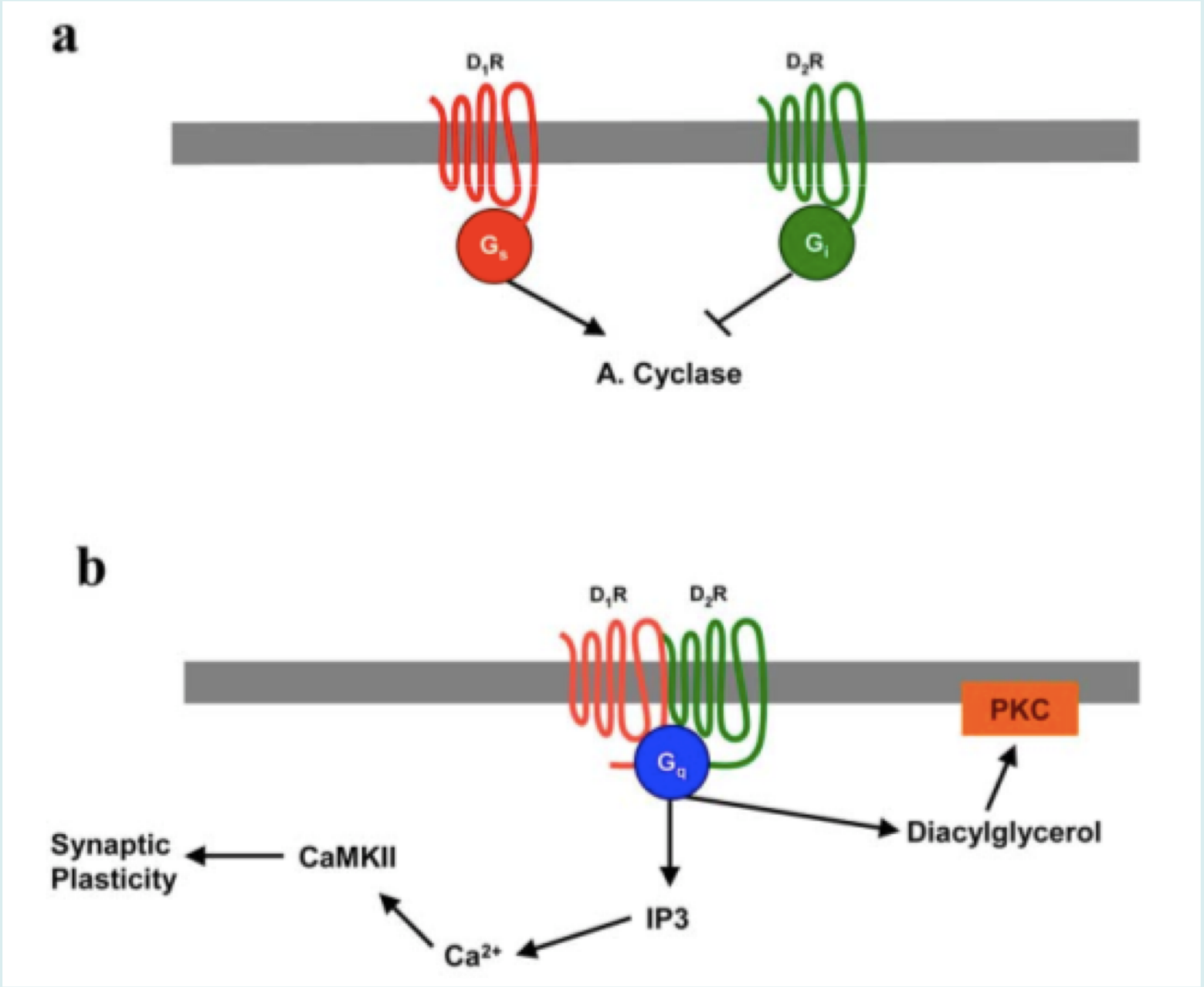


FIGURE 3. Intramolecular interactions in the receptor heterodimer. The affinity of a given ligand for its receptor (central drawing) may decrease (a) or increase (b) when the receptor forms heterodimers and also may be decreased (c) or increased (d) when the receptor partner (in the heterodimer) is occupied by the same (for isoreceptors) or another ligand.

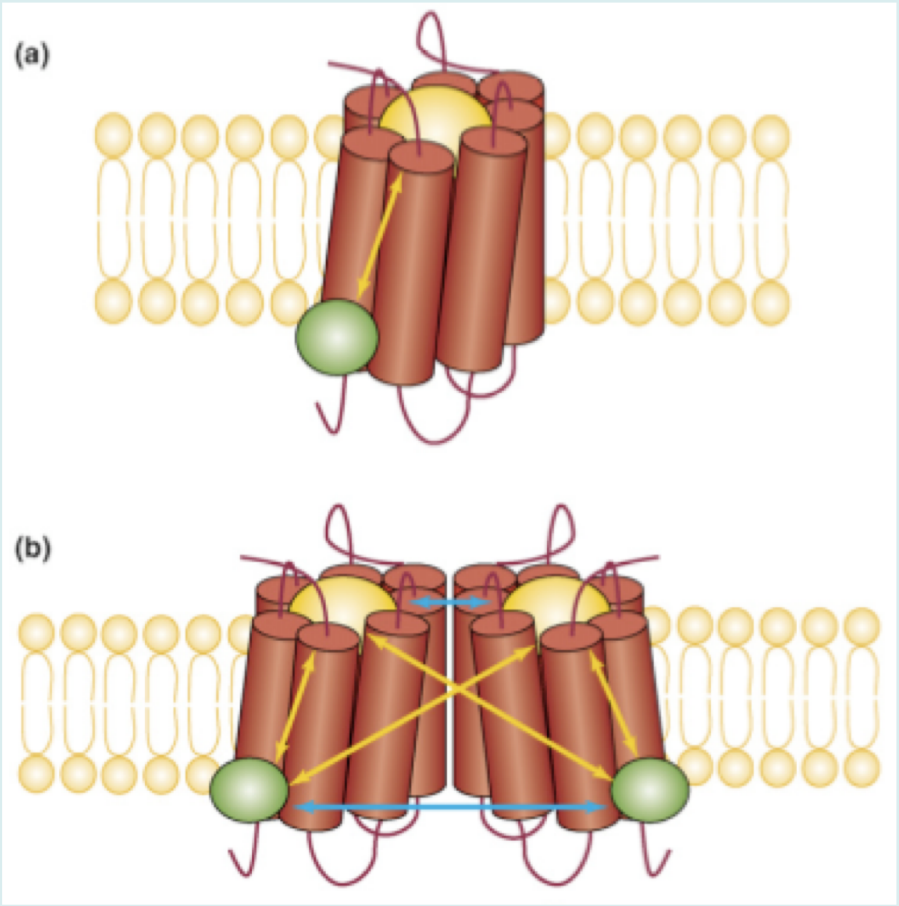
Heterodimerization - change in binding

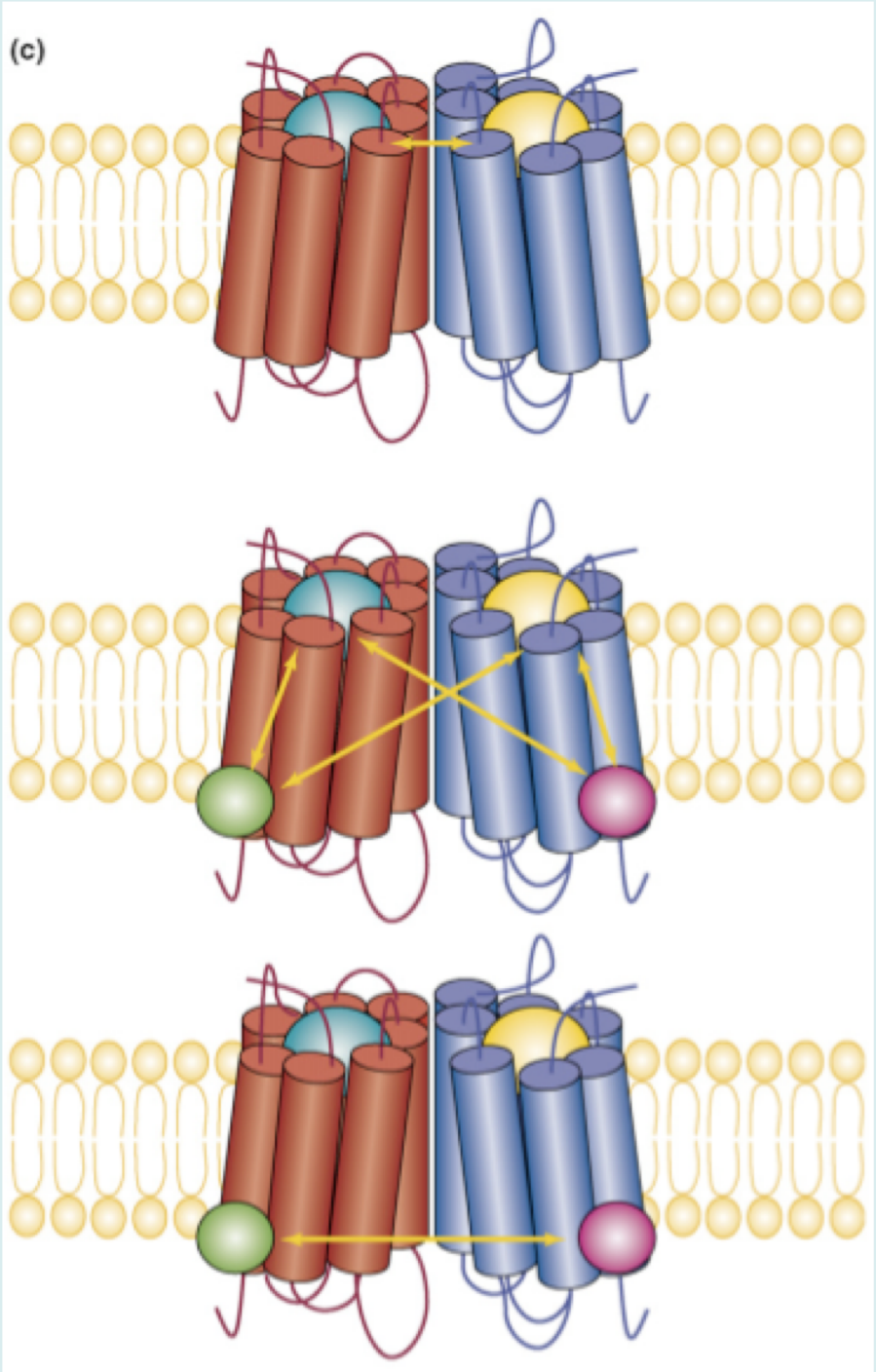


Heterodimerization - change in coupling

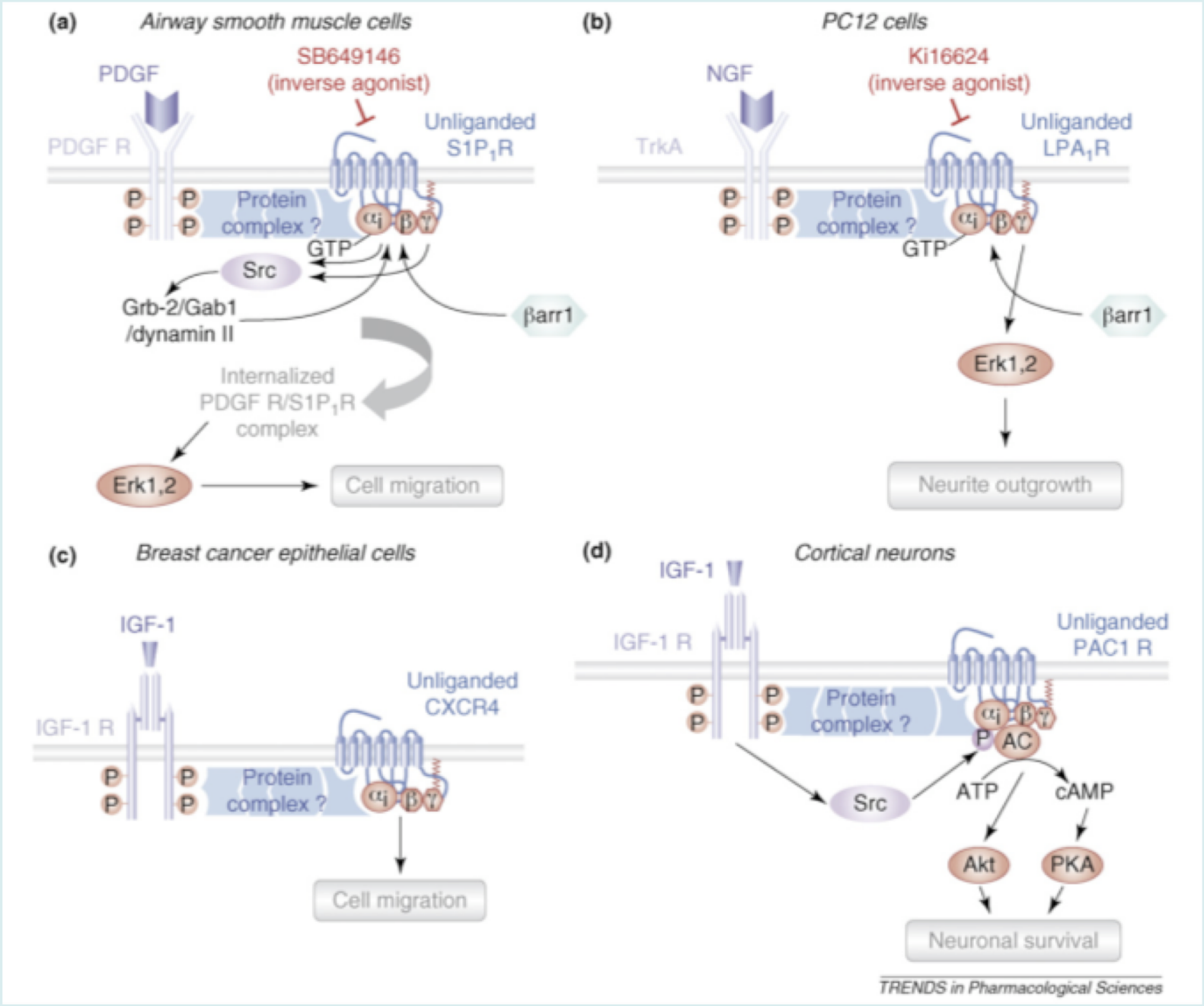


Allostery in dimers

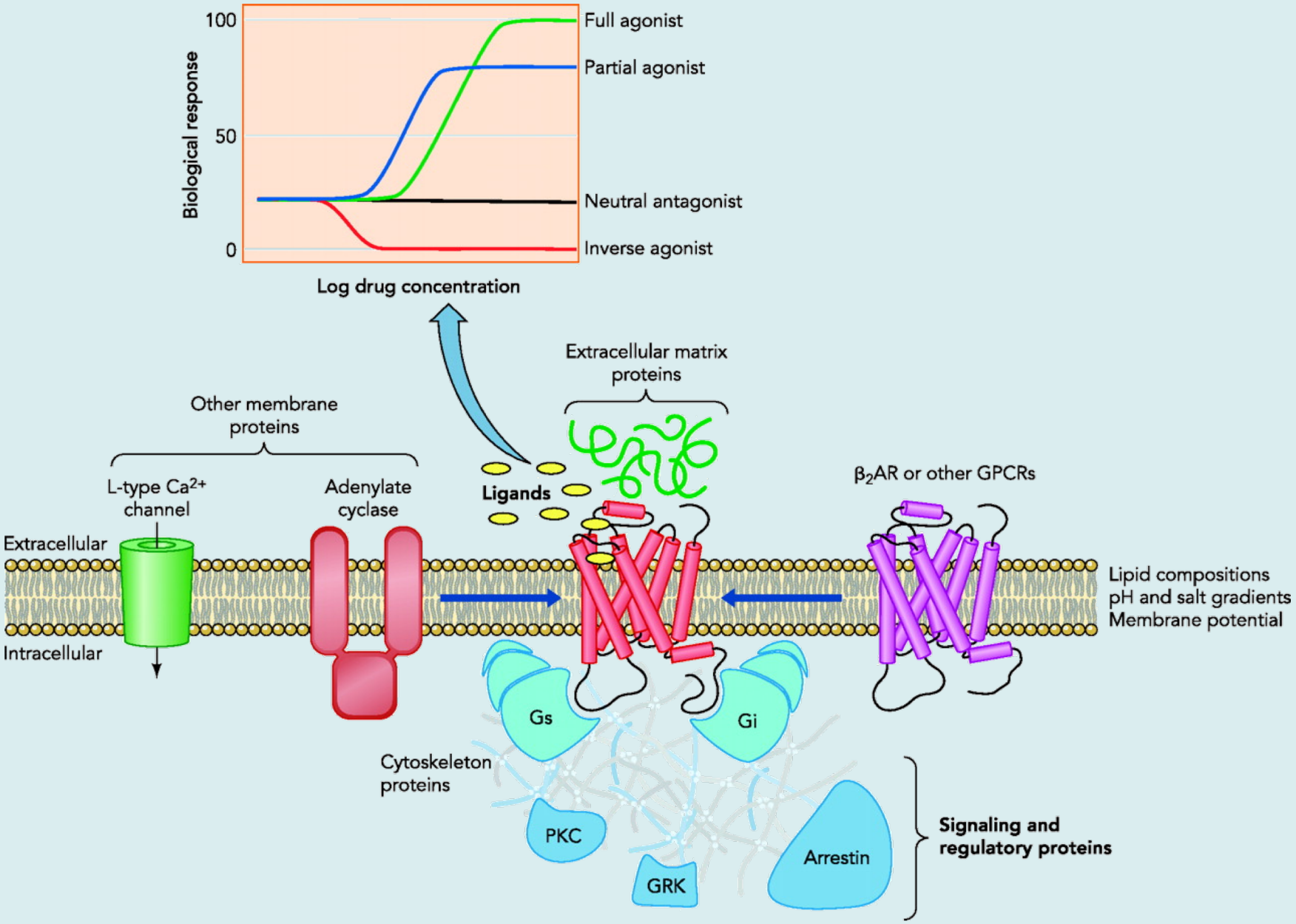




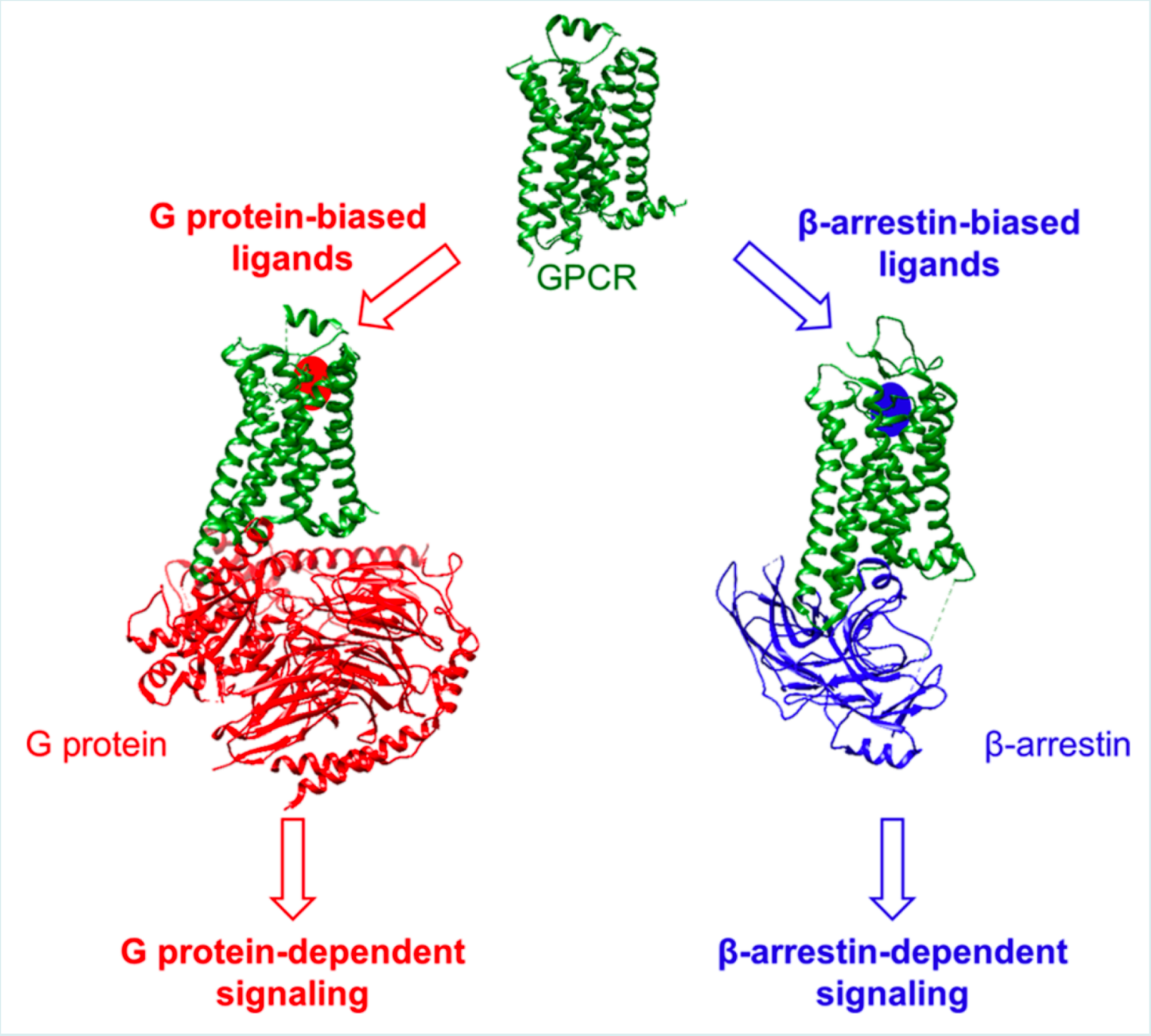
Transactivation of GPCR by RTK



GPCR signaling: intertaction network

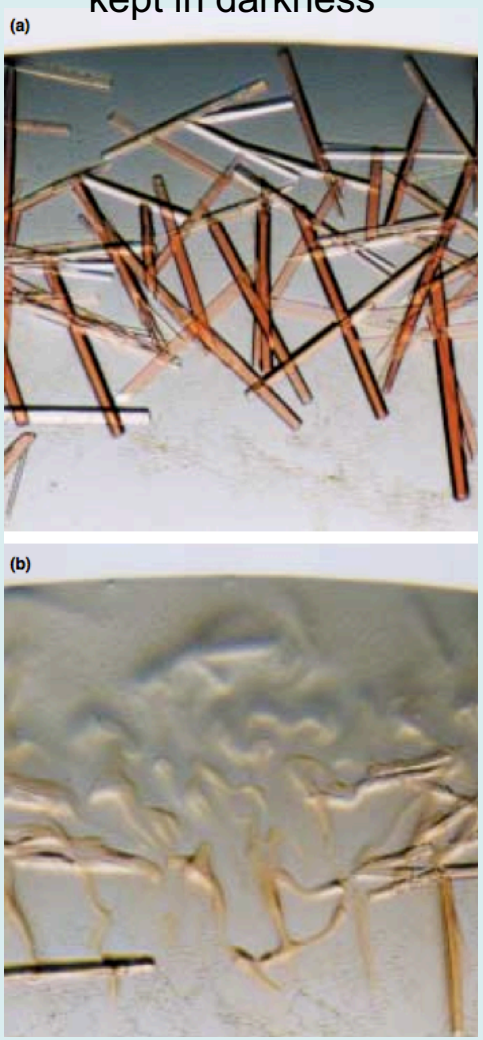


Biased agonism of GPCR

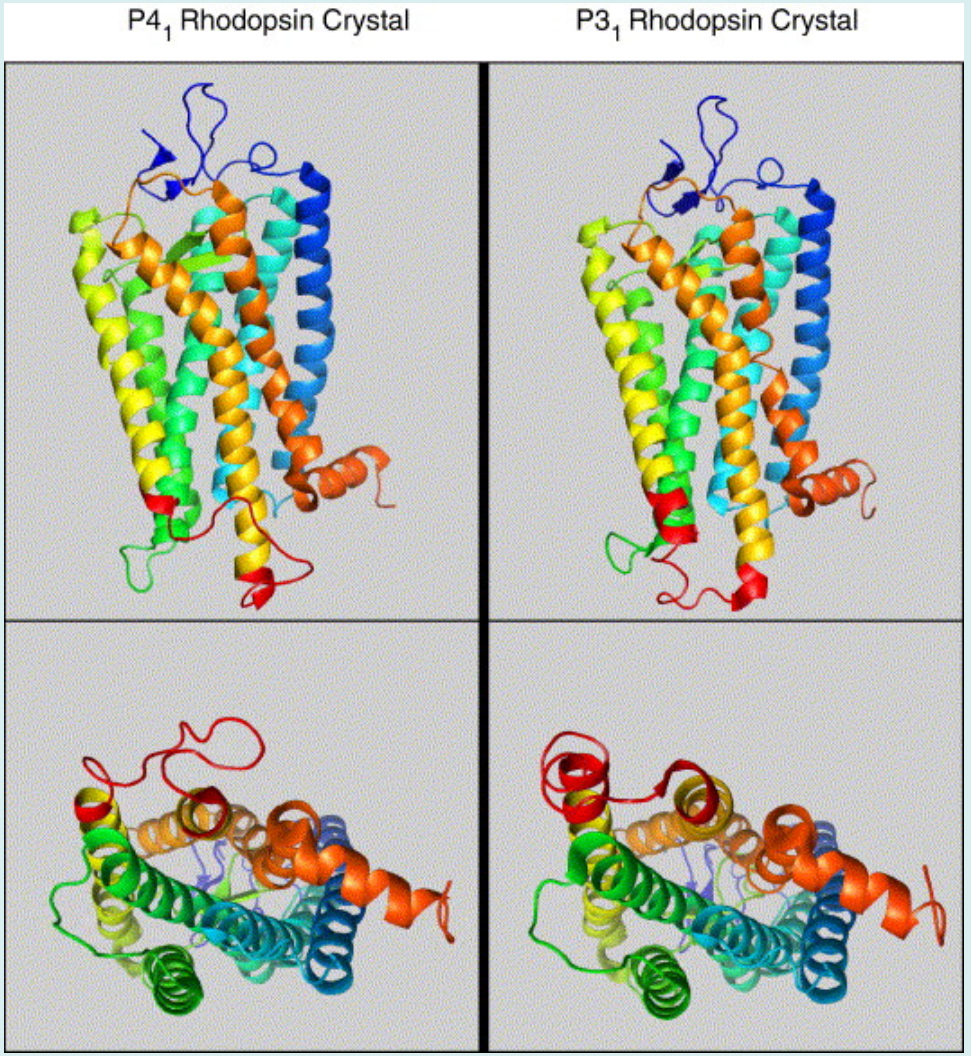


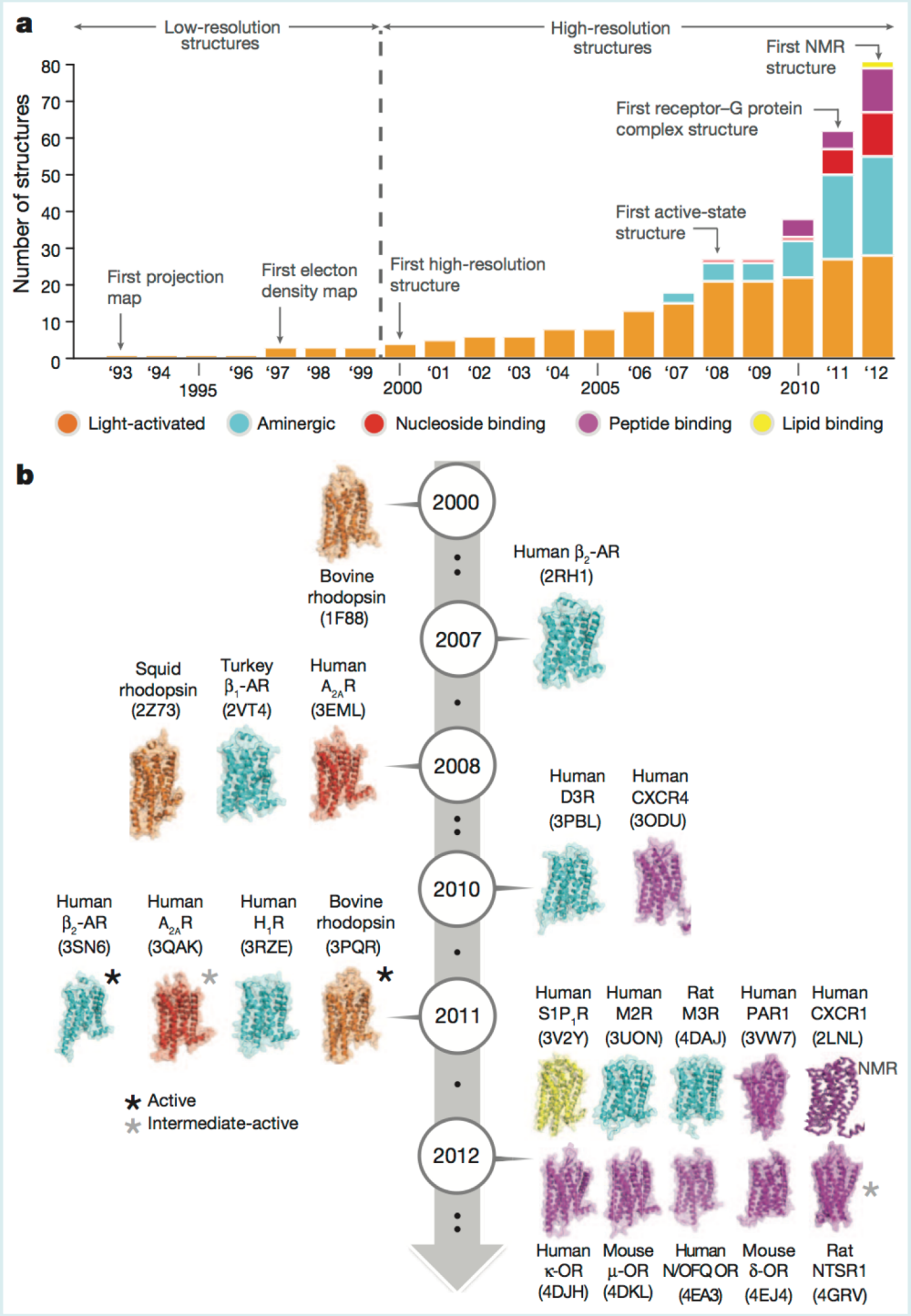
The first crystal structure: Rhodopsin

kept in darkness



in light




a
Low-resolution structures
High-resolution structures
First NMR structure
First receptor–G protein complex structure
First active-state structure
First projection map
First electon density map
First high-resolution structure
Number of structures
80
70
60
50
40
30
20
10
0
'93 '94 1995 '96 '97 '98 '99 2000 '01 '02 '03 '04 2005 '06 '07 '08 '09 2010 '11 '12
Light-activated
Aminergic
Nucleoside binding
Peptide binding
Lipid binding
b
2000
Bovine rhodopsin (1F88)
Human β2-AR (2RH1)
2007
Squid rhodopsin (2Z73)
Turkey β1-AR (2VT4)
Human A2AR (3EML)
2008
Human D3R (3PBL)
Human CXCR4 (3ODU)
2010
Human β2-AR (3SN6)
Human A2AR (3QAK)
Human H1R (3RZE)
Bovine rhodopsin (3PQR)
2011
Human S1P1R (3V2Y)
Human M2R (3UON)
Rat M3R (4DAJ)
Human PAR1 (3VW7)
Human CXCR1 (2LNL)
NMR
2012
Human κ-OR (4DJH)
Mouse μ-OR (4DKL)
Human N/OFQ OR (4EA3)
Mouse δ-OR (4EJ4)
Rat NTSR1 (4GRV)
Active
Intermediate-active

Crystal structure of active-state GPCR

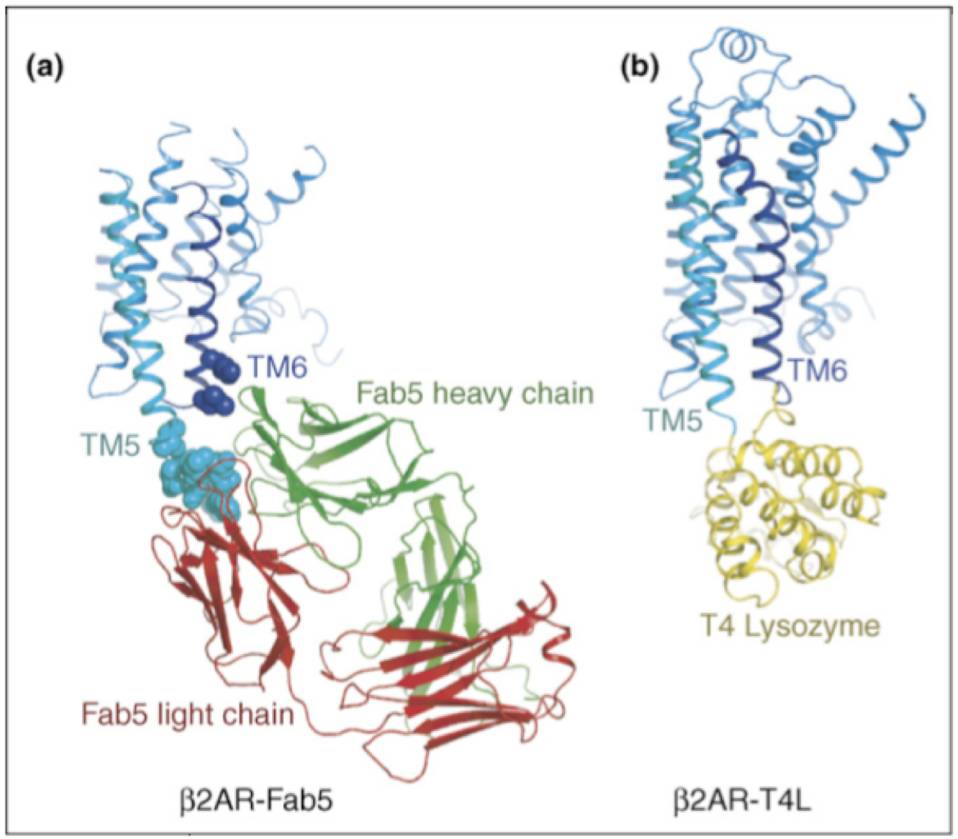
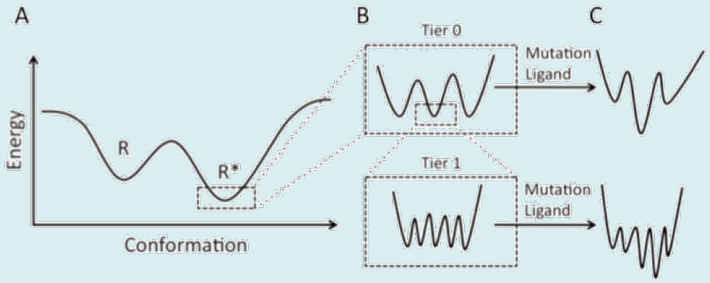
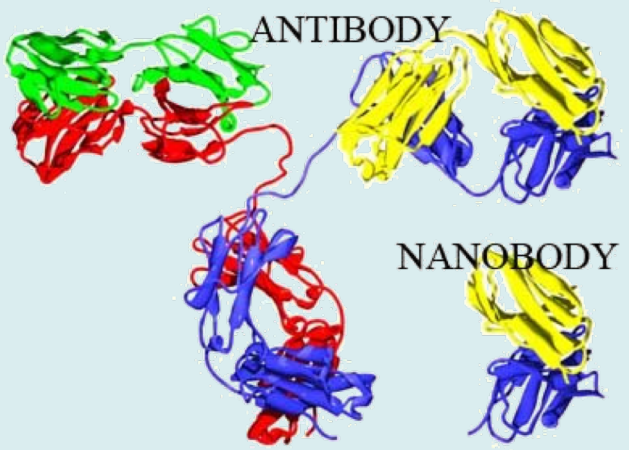


Figure 1. Structures of the β_2AR. **(a)** Wild-type β_2AR in complex with Fab5. **(b)** Engineered β_2AR-T4-lysozyme fusion protein.TM, transmembrane segment; ICL2, second intracellular loop. Fab5 and T4 lysozyme serve similar functions in these two crystal structures. They both stabilize interactions between TM5 and TM6, and provide additional polar surface area for crystal lattice contacts.



High conformational flexibility – fragile crystals.

The active state must be stabilized – fusion proteins, IgG fragments, nanobodies



Homology modeling and ligand docking from a pharmacological point of view

What we need for rational drug design is 3D-structure!

In order to design ligands selective to a given receptor conformer (agonist or antagonist or inverse agonist), detailed knowledge of the 3D-structure of the receptor or at least its ligand binding site is necessary.

Computational models may help drug discovery.

Techniques of determining the ligand binding pocket of GPCR

- Alanine-scanning mutagenesis
- Substituted cysteine accessibility method
- Photoaffinity crosslinking
- **Computational modeling-guided mutagenesis**

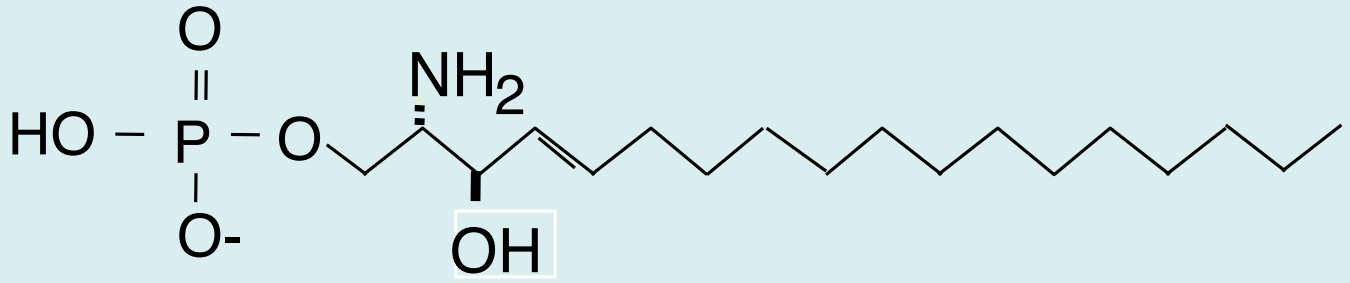
3D-structure “estimation” by homology modeling

- Sequence alignment of the protein of interest to the one with known tertiary structure
- Arrangement of the positions of amino acids as it was in the known structure
- Energy minimization of the new structure to find possible conformation of the protein
- Manual refinement to remove gaps in the TM domain and find interhelical interactions (H-bond network)
- Optimization of the structure by molecular dinamics simulation followed by energy minimization
    - ***An example: lysophospholipid receptors***

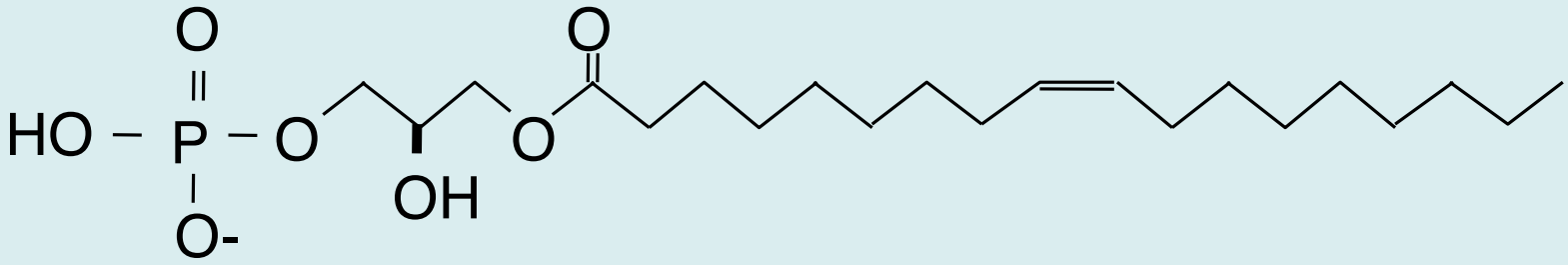
Lysophospholipid Mediators Regulate Important Physiological Processes

- Regulation of cell cycle - promoting proliferation
- Cell survival factors - inhibiting apoptosis
- Actions on cell motility by cytoskeleton rearrangement
- Effects on differentiation, cell-cell and cell-matrix communication
- Influencing intracellular calcium homeostasis

Two Classes of Lysophospholipid Mediators:
Nature's simplest sphingo- and glycerophospholipids

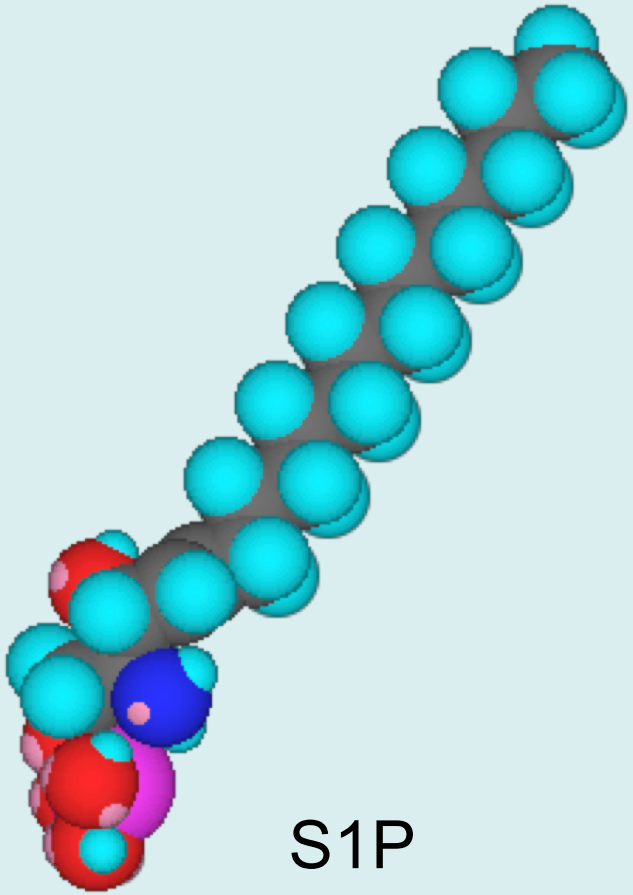


Sphingolipid mediator: sphingosine-1-phosphate (S1P)

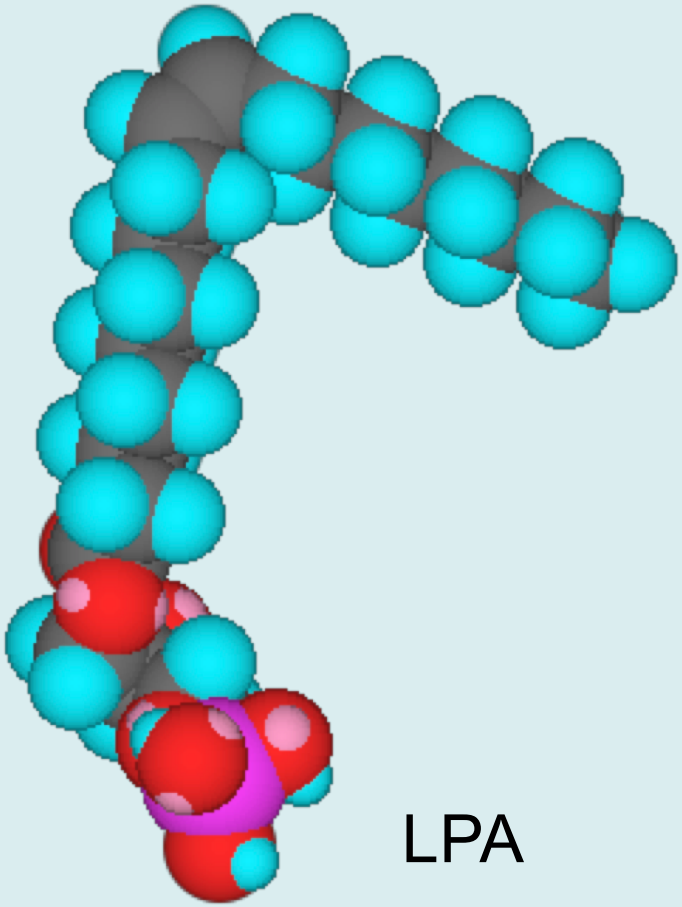


Glycerolipid mediator: lysophosphatidic acid (LPA)

Space-Filling Models of the Prototypic Lysophospholipid Mediators

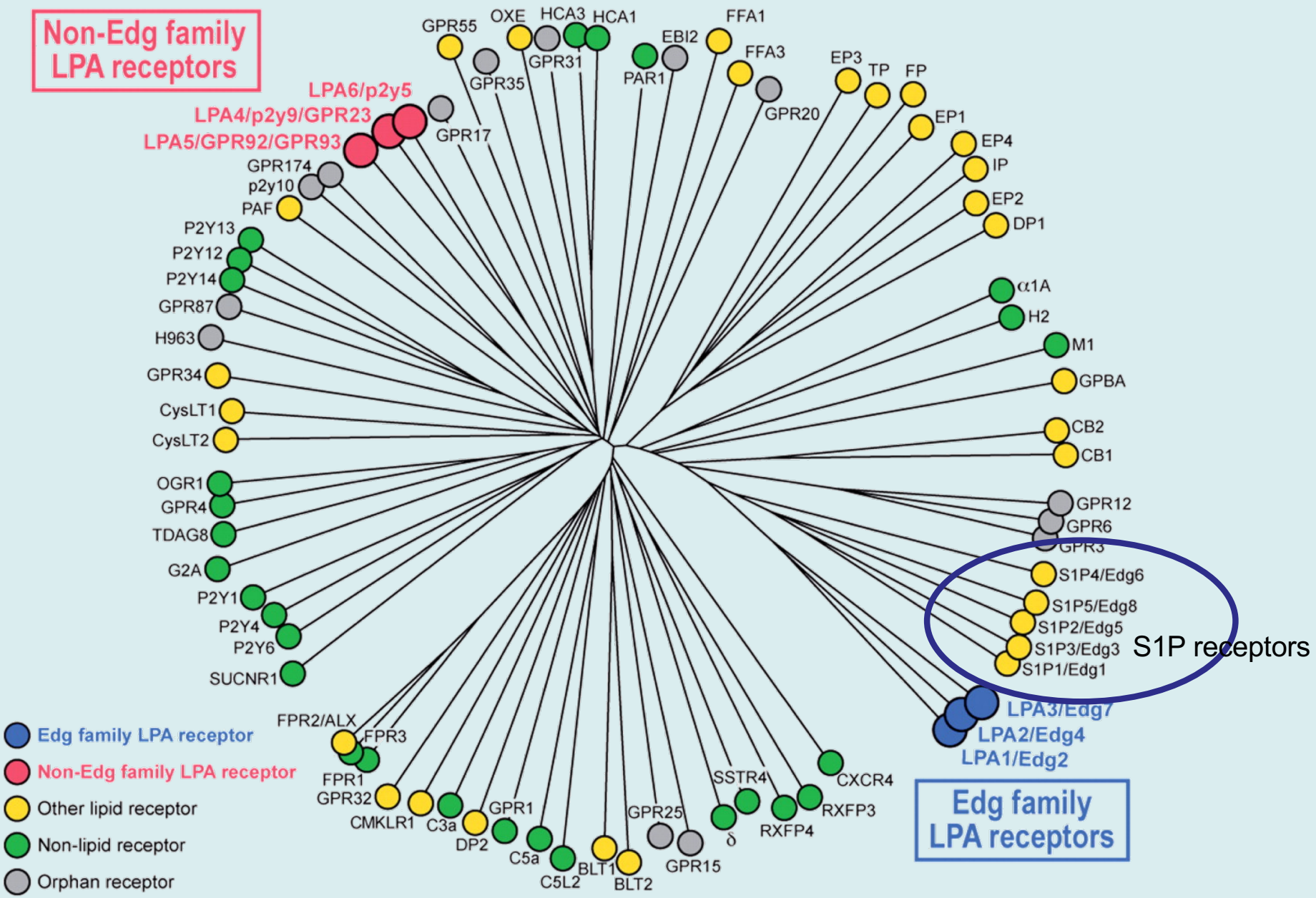


S1P

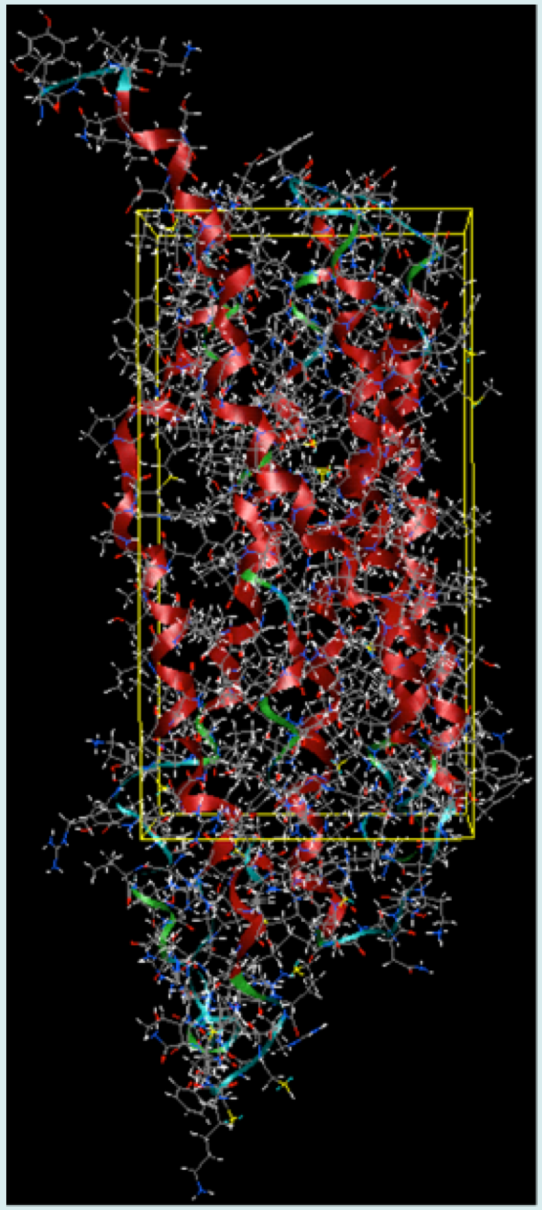


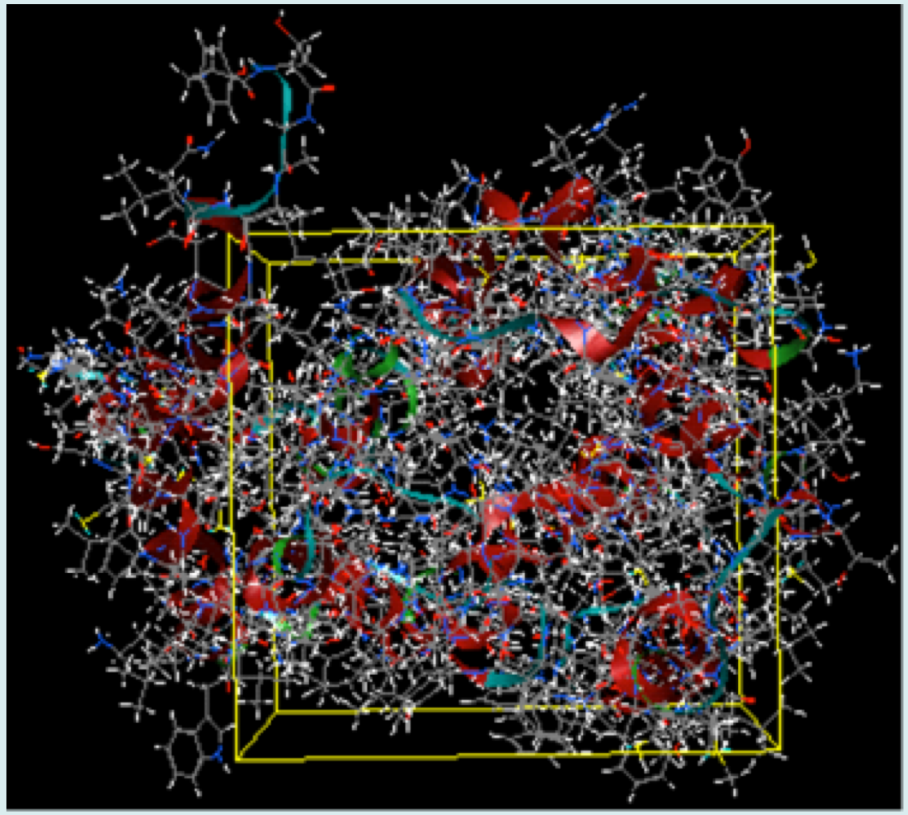
LPA

Identification of Lysophospholipid Receptors



Homology model of $S1P_1$

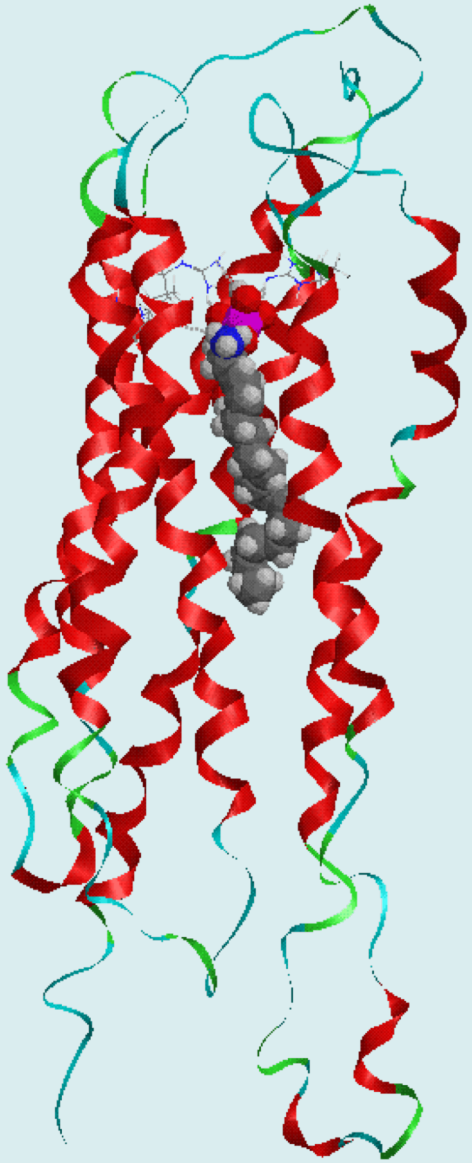




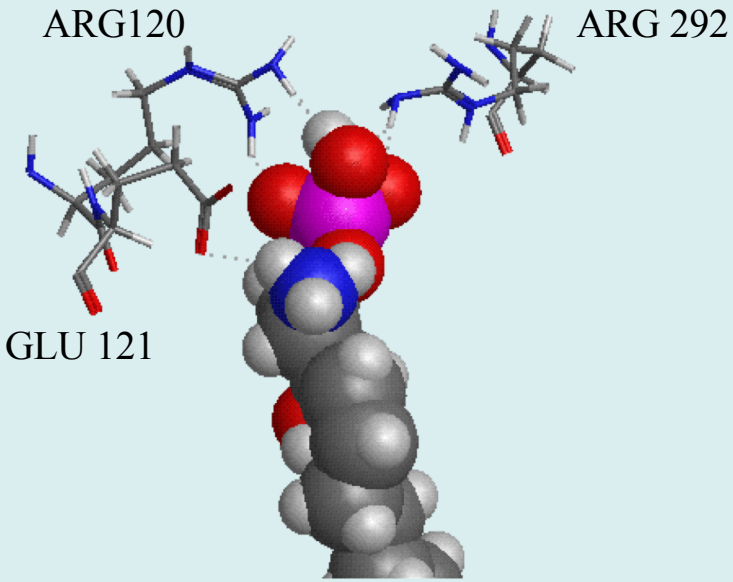
Ligand-receptor Complex Modeling

- Docking volume is defined manually
- Calculations of steric- and electrostatic-interactions with the protein at each point at an equally-spaced grid within the docking box from random starting positions (protein has no flexibility)
- Geometry optimization with AMBER94 molecular mechanics force-field program (protein is allowed minimal flexibility to optimize local energy minimum in the vicinity of the ligand on the potential energy surface)

$S1P_1$ Complex with S1P



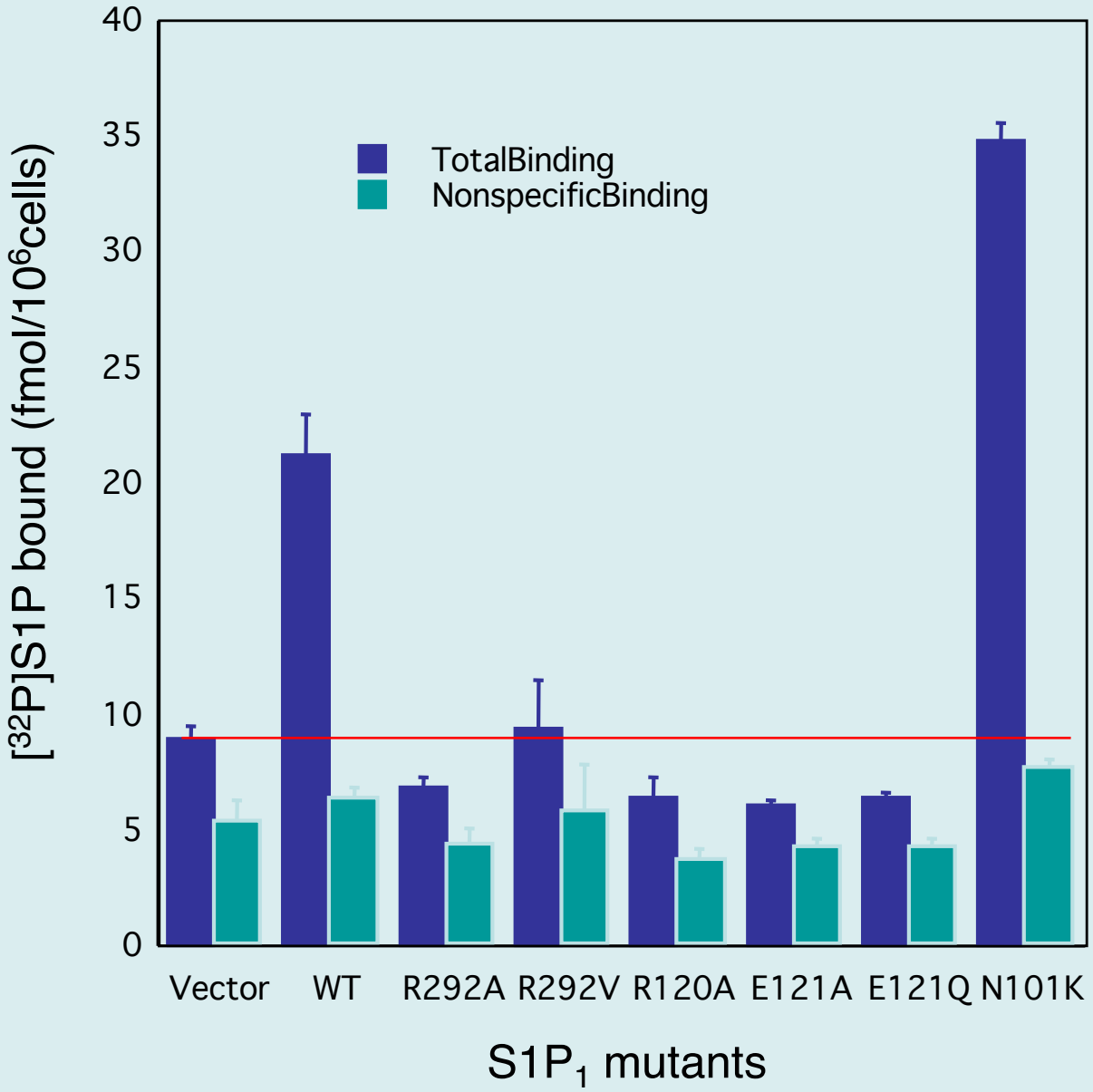
- Phosphate forms ion pairs with ARG 120 and 292
- Amine forms ion pair with GLU 121



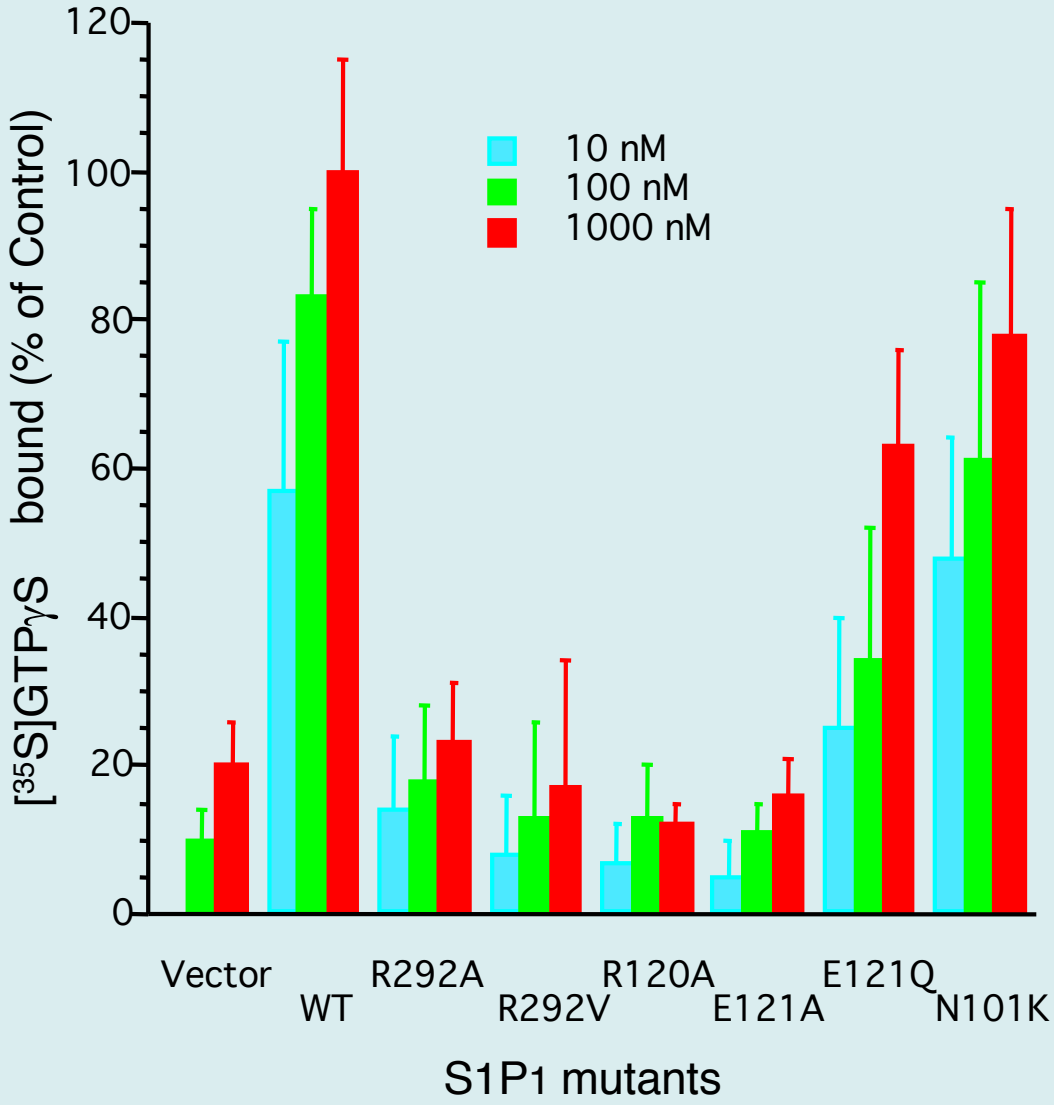
- Computational modeling can provide valuable information regarding the 3D-structure of GPCR and their complexes with ligands – but…

- **Predictions from computational modeling must be verified experimentally, e.g. by site-directed mutagenesis and functional measurements!**

Ligand binding: S1P Binding to $S1P_1$ Mutants

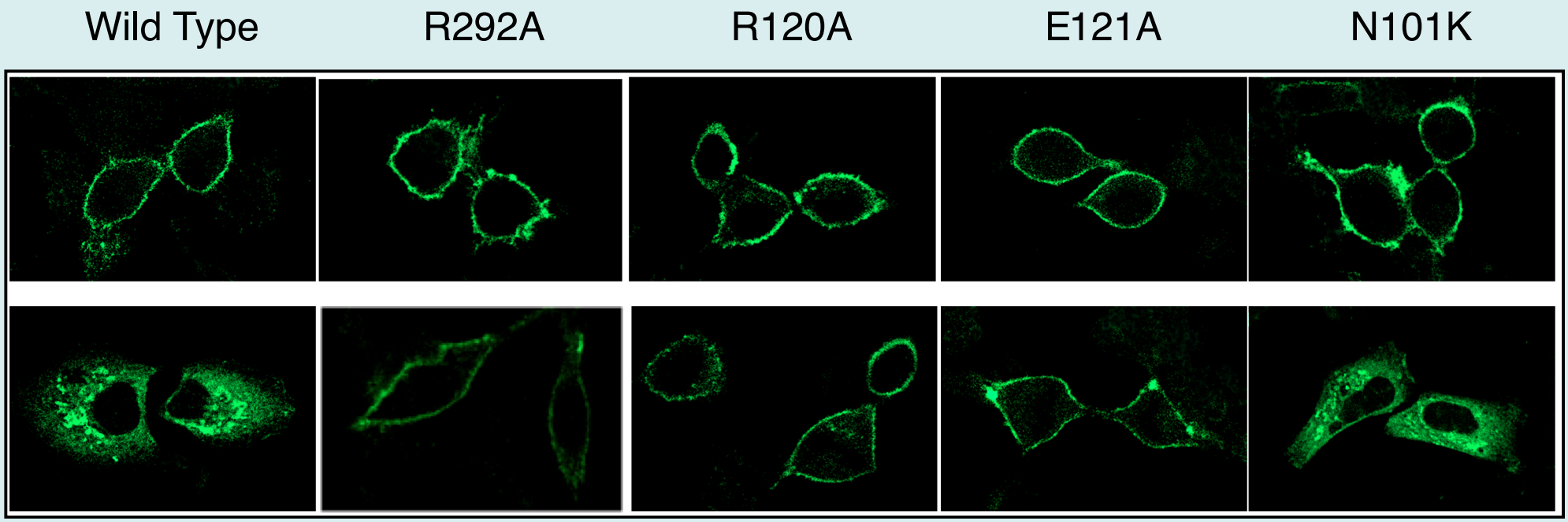


Ligand-elicited [^{35}S]GTPγS Binding to S1P$_1$ Mutants



	EC $_{50}$
Vector	N.D.
Wild Type	8.5 ± 1.4 nM
R292A	22 mM
R292V	14 mM
R120A	24 mM
E121A	900 nM
E121Q	320 ± 90 nM
N101K	15.4 ± 1.8 nM
K111A	19.1 ± 2.9 nM

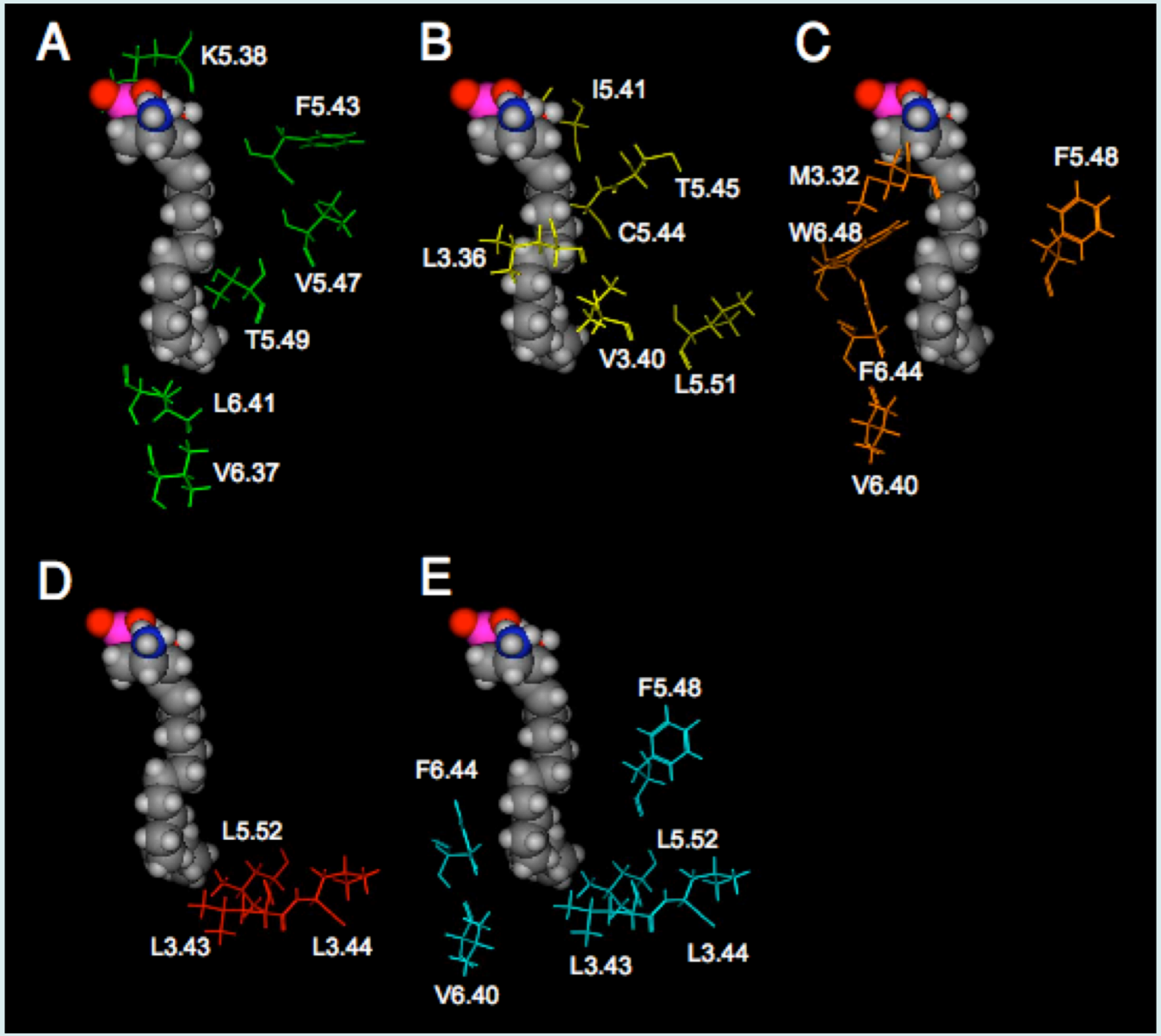
Ligand-induced Internalization of $S1P_1$ Receptor Mutants



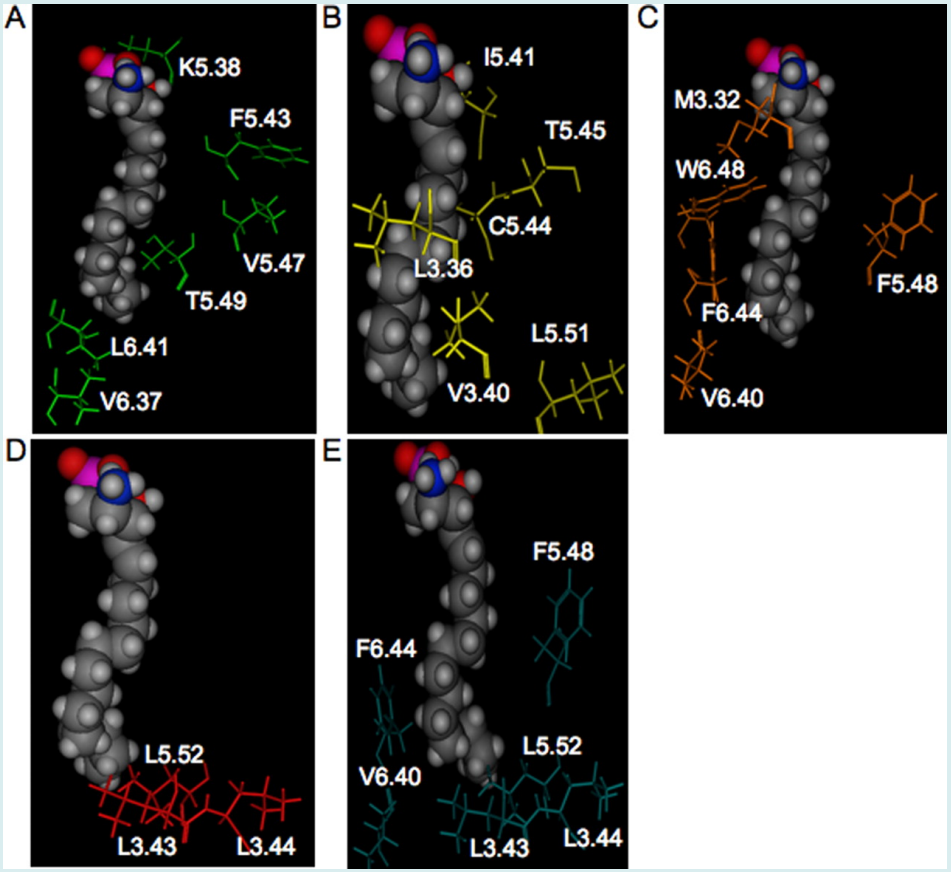
Upper panel: Non-stimulated

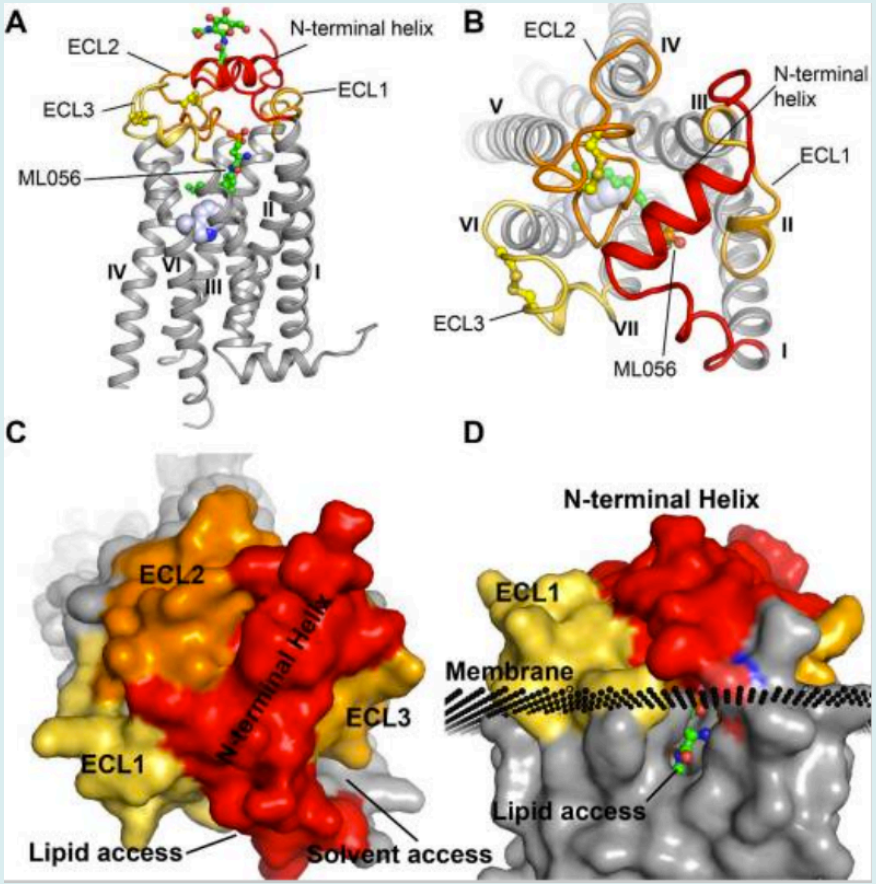
Lower panel: 100 nM S1P, 15 min

Mapping the hydrophobic part of the ligand binding pocket in $S1P_1$



$S1P_1$ crystal structure has been determined...

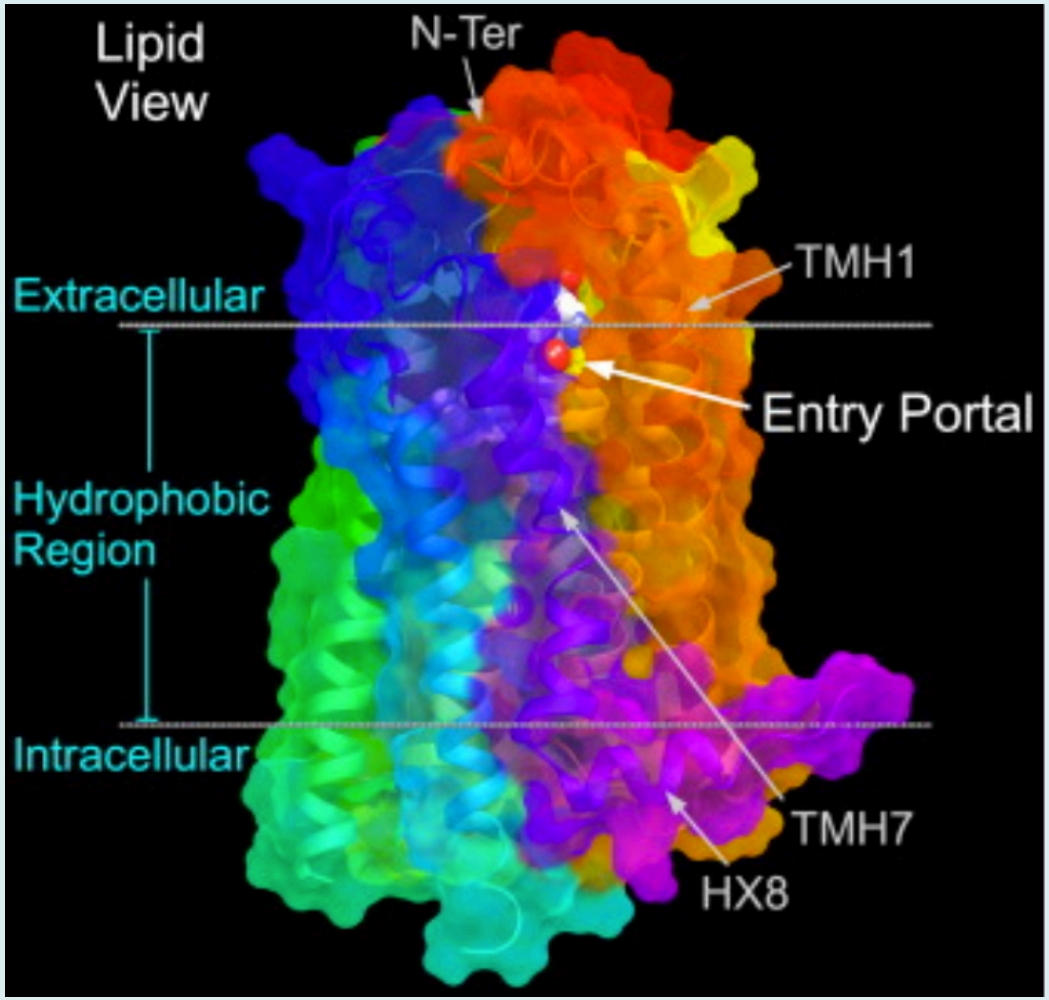




- Fujiwara Y, Osborne DA, Walker MD, Wang DA, Bautista DA, Liliom K, VanBrocklyn JR, Parrill AL, Tigyi G. Identification of the hydrophobic ligand binding pocket of the S1P1 receptor. J Biol Chem. (2007) 282(4):2374-85.

- Hanson MA, Roth CB, Jo E, Griffith MT, Scott FL, Reinhart G, Desale H, Clemons B, Cahalan SM, Schuerer SC, Sanna MG, Han GW, Kuhn P, Rosen H, Stevens RC. Crystal structure of a lipid G protein-coupled receptor. Science. (2012) 335(6070):851-5.

$S1P_1$ crystal structure has been determined...



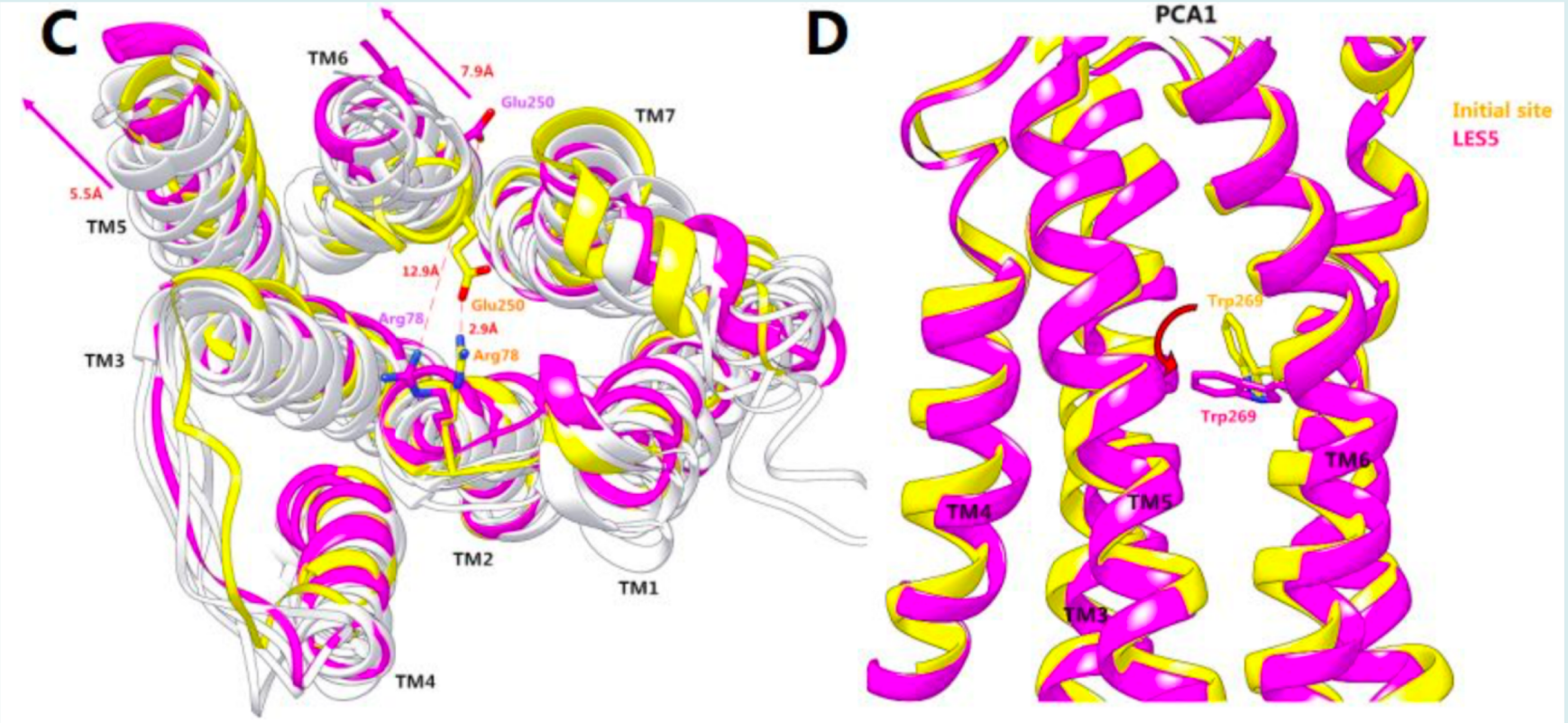
The natural agonist lipid S1P can enter the receptor only from the outer leaflet of the plasma membrane.

A Computational Approach to the Study of the Binding Mode of S1P1R Agonists Based on the Active-like Receptor Model

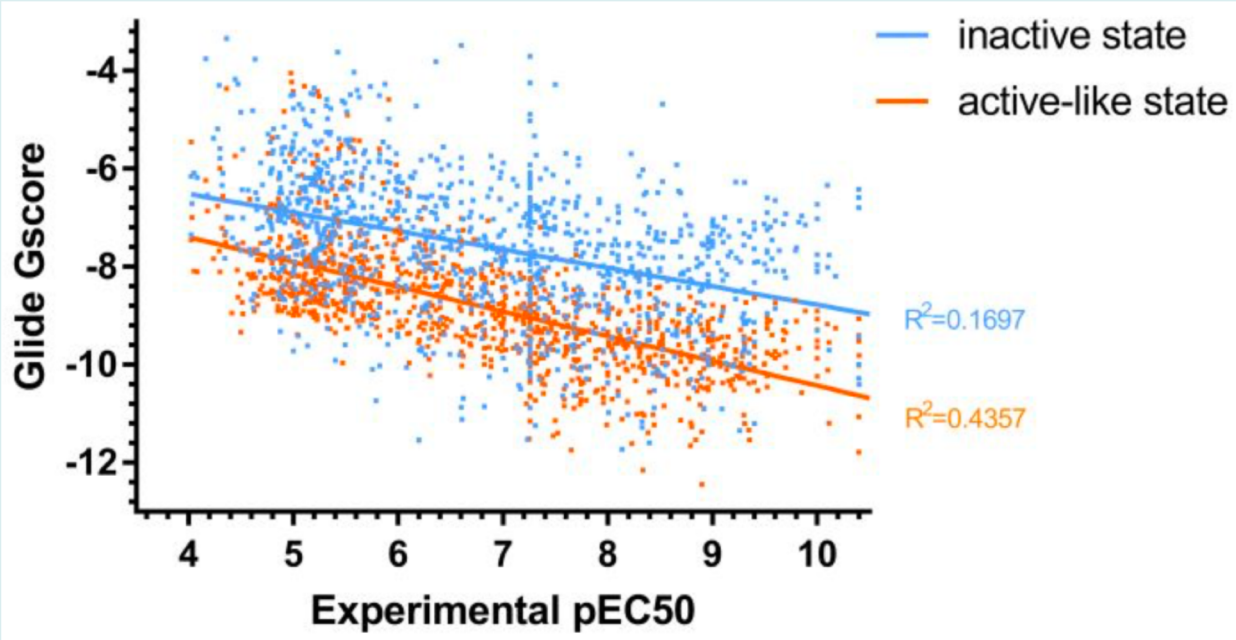
Yonghui Chen, Tianqi Liu, Qiumu Xi, Wenqiang Jia, Dali Yin, and Xiaojian Wang

J. Chem. Inf. Model., **Just Accepted Manuscript** • DOI: 10.1021/acs.jcim.8b00764
Publication Date (Web): 11 Mar 2019

novel approach: Gaussian accelerated molecular dynamics

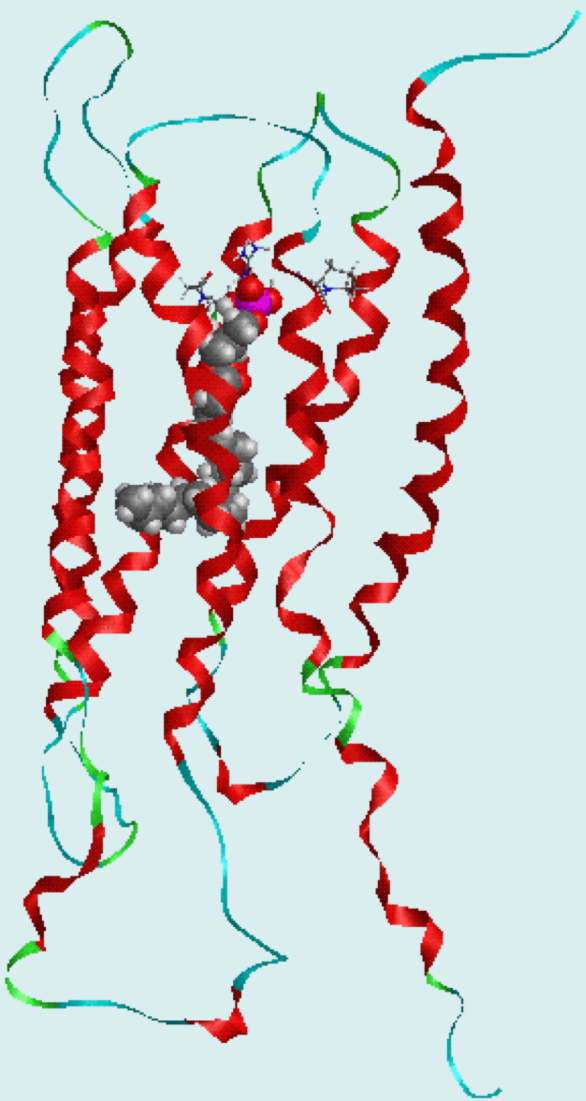


Validation by docking

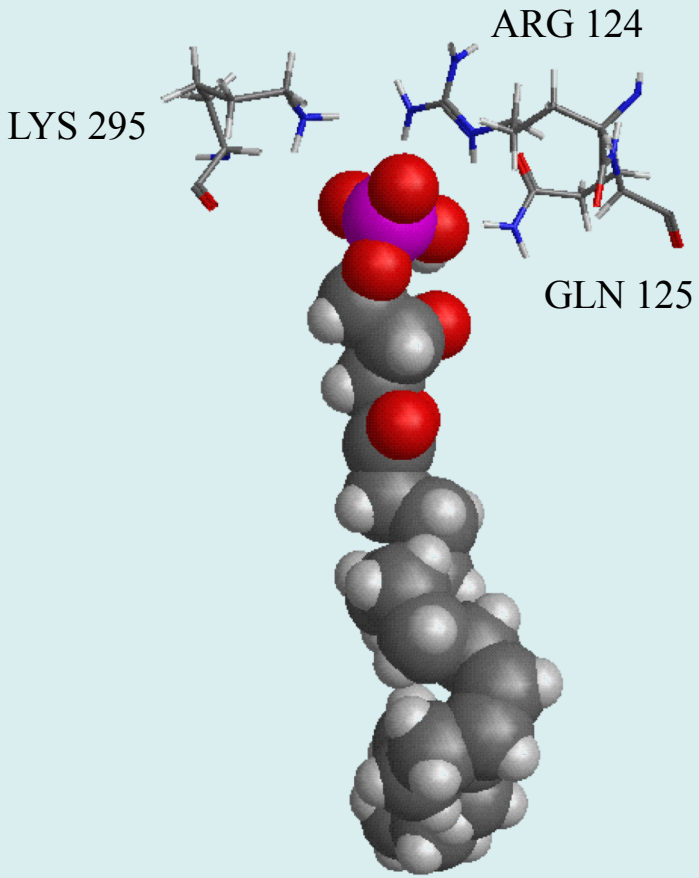


The experimentally validated model can be used for homology-modeling of closely related GPCR

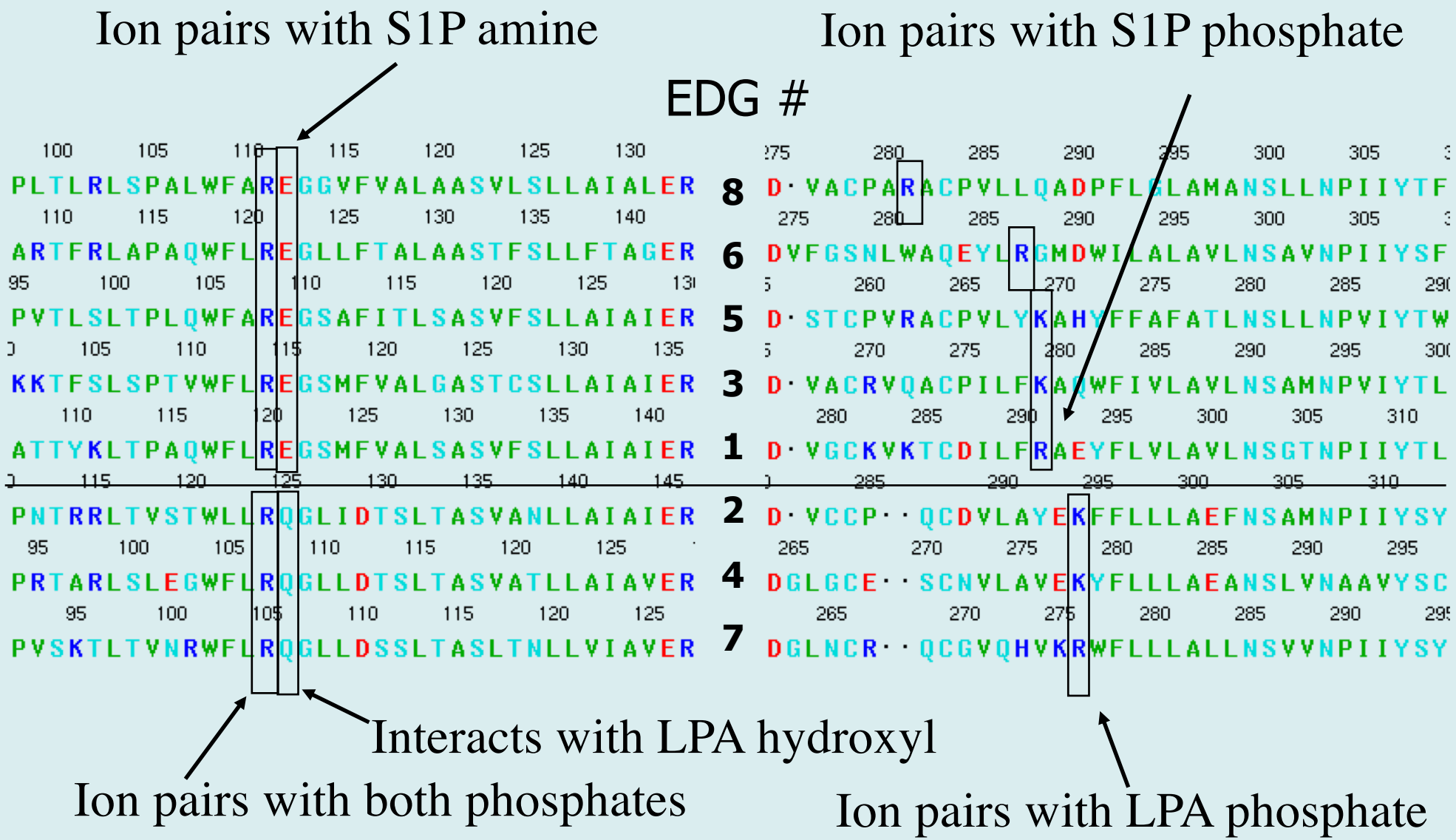
LPA_1 Complex with LPA



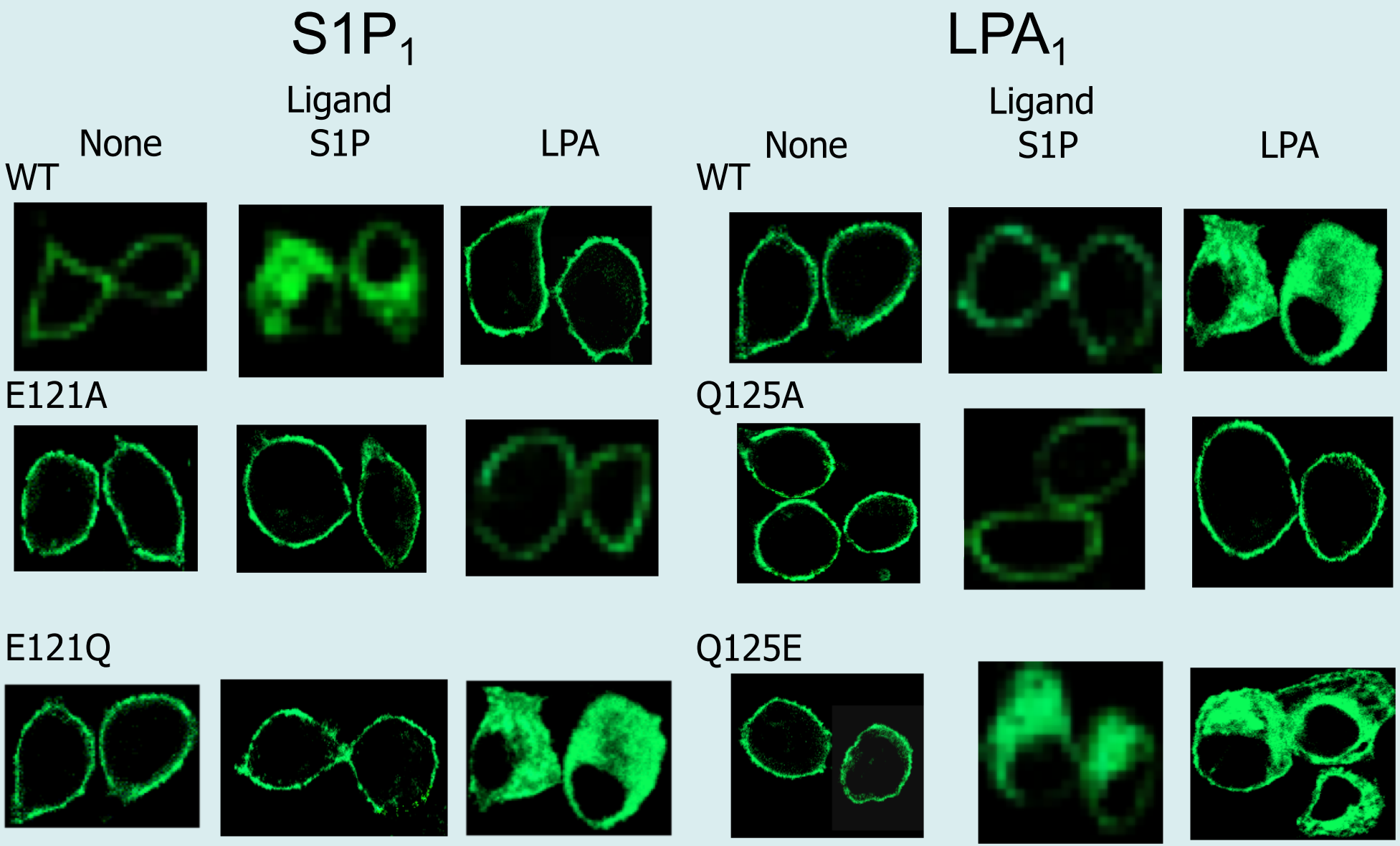
- Phosphate forms ion pairs with ARG 124 and LYS 295
- Hydroxyl interacts with GLN 125



Residues predicted to determine S1P/LPA selectivity



Ligand Specificity Reversal



Brief Summary

- GPCR are general environment-sensors with heptahelical plasmamembrane domain arrangement and common structural and signaling mechanisms
- GPCR are unique - they have basal activity in the absence of their ligands and this constitutive activity can be enhanced (agonist) or attenuated (inverse agonist) by a ligand
- For effective drug development we need to know the 3D-structure of the ligand binding pockets - homology modeling to the X-ray structure of rhodopsin
- The predicted structure of ligand-GPCR complexes has be to validated experimentally: ligand binding and functional studies on mutant receptors in which the key interactions are eliminated